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(57) Abstract

An essentially purified preparation of human Lung Cancer-associatted Protein (LCAP), a monoclonal antibody specific for LCAP, and a method of detecting LCAP in a biological sample, which method includes the steps of (1) contacting the biological sample with an aliquot containing the monoclonal antibody specific for LCAP, and (2) detecting immune complex formation between the antibody and a constituent of the biological sample, such immune complex formation being indicative of the presence of LCAP in the biological sample.

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LUNG CANCER-ASSOCIATED PROTEIN

Background of the Invention

The field of the invention is immunoassays for cancer-specific antigens.

Many types of cancer have been found to be associated with increased circulating levels of particular biochemical markers. For example, markers termed DF3 antigen (Abe and Kufe, J. Immunol. 139:257-61, 1987) and carcinoembryonic antigen, or CEA (Shively and Beatty, CRC Crt. Rev. Oncol/Hematol. 2:344-399, 1985), have been found to be present at statistically higher levels in the serum of patients with breast cancer than in the serum of normal individuals, while squamous cell carcinoma-associated (SCC) antigen, calcitonin, ferritin, glycosyl transferase, and CEA have been described as possible circulating marker candidates for lung cancer (see references cited in Maimonis et al., Cancer Research 51:3838-3842, 1991, which is herein incorporated by reference). Lung cancers can be divided into two groups: small-cell carcinomas and non-small-cell carcinomas, the latter category including epidermoid or squamous cell carcinomas, adenocarcinomas, and large cell carcinomas. Serial determinations of the circulating levels of an appropriate biochemical marker are useful for monitoring the clinical course of a given cancer patient's disease.

Summary of the Invention

In general, the invention features an immunoassay for an antigen termed Lung Cancer-associated Protein, or LCAP. This antigen has been found to be expressed on the surface of human lung cancer cells, both from primary tumors and from lung cancer-derived cell lines (Maimonis et al., Cancer Research 50:6738-6743, 1990, herein incorporated by reference), and is also found in the

serum of lung cancer patients at levels significantly higher than those for normal individuals (Maimonis et al., Cancer Research 51:3838-3842, 1991, herein incorporated by reference). The immunoassay of the invention provides a method for detecting LCAP in a biological sample (e.g., blood, serum, urine, sputum, mucosal scrapings, or biopsied tissue from a human or another mammal), which method includes the steps of contacting the biological sample with an aliquot or sample containing a monoclonal antibody (MAb) specific for LCAP, and detecting immune complex formation between the antibody and a constituent of the biological sample (for example, by ELISA), such immune complex formation being indicative of the presence of LCAP in the biological sample. The method of the invention may include the additional steps of providing a control sample containing a standard amount of LCAP (either purified or in a mixture, such as a sample of serum containing a known amount of LCAP); contacting the control sample with a second aliquot containing the monoclonal antibody; and comparing the amount of immune complex formation in the biological sample to the amount of immune complex formation in the control sample. A monoclonal antibody specific for LCAP is herein defined as an antibody which (1) forms an immune complex with LCAP antigen purified from the supernatant of cultured CALU-3 cells (American Type Culture Collection accession no. ATCC HTB 55), and (2) detects circulating antigen at elevated levels (i.e., above the normal cutoff level of 23 units of LCAP/ml) with serum samples from at least 70% of patients with either adenocarcinoma or squamous cell carcinoma of the lung; one such MAb is that produced by the hybridoma DF-L1.

Also within the invention is an immunoassay kit including (1) a first reagent including a first

monoclonal antibody specific for LCAP; (2) a second reagent including an enzyme conjugated to a second monoclonal antibody specific for LCAP (which second antibody may be identical to the first antibody, or at least capable of binding to the same type of determinant as that bound by the first antibody); a third reagent which includes a substrate for the enzyme; and instructions for using the kit. One or both of the monoclonal antibodies may optionally be that produced by the hybridoma DF-L1, or may be a MAb which binds to the same determinant as that bound by the MAb produced by the hybridoma DF-L1. The enzyme and substrate are preferably horseradish peroxidase and hydrogen peroxide, respectively. The kit may also include a fourth reagent that includes LCAP, to be used, for example, as a calibrator or control sample. This fourth reagent may be, e.g., a sample of human serum containing a predetermined amount of LCAP, or may contain essentially purified LCAP in dry form or in solution. In order to obtain an essentially purified preparation of LCAP (herein defined as a preparation containing LCAP essentially [i.e., greater than 95%] free of the human proteins with which it is naturally associated on cells or in bodily fluids such as serum), the antigen may be extracted from membranes of human cells (e.g., primary tumor cells or a cell line expressing LCAP), or isolated from a bodily fluid (such as blood) or the medium bathing an *in vitro*-cultured cell which secretes LCAP. An essentially purified preparation of LCAP may be made by (1) providing a population of cells capable of expressing LCAP (e.g., on their membranes and/or secreted into the medium bathing the cells); (2) culturing the population of cells in a medium under conditions which permit the population of cells to express LCAP; and isolating LCAP from the membranes of the cells, or from the medium [for

example, by contacting the membranes, an extract of the membranes, or the spent medium with an immunoaffinity matrix having an antibody specific for LCAP (such as the MAb produced by the hybridoma DF-L1) affixed to a matrix material]. The population of cells is preferably descended from a CALU-3 cell (ATCC HTB 55), and is preferably cultured in a medium containing at least 50 μg/ml (more preferably 100 to 300 μg/ml, and most preferably 150 μg/ml) galactosamine. The invention also includes a monoclonal antibody specific for LCAP, a hybridoma cell which expresses such an antibody, and a method of producing the antibody, including the steps of culturing the hybridoma cell in a medium and isolating the antibody from the medium.

In another aspect, the invention includes an immunotoxin in which the antibody portion of the immunotoxin is an LCAP-specific monoclonal antibody (e.g., the MAb produced by hybridoma DF-L1), or an LCAP-binding fragment thereof, conjugated to a toxin molecule. Such conjugation may be accomplished by known chemical methodology, or, if the toxin is a protein, by means of genetically engineering a hybrid DNA molecule encoding both the toxin and an LCAP-binding portion of the antibody as a single polypeptide: expression of this recombinant DNA molecule would result in an immunotoxin in which the antibody portion is linked to the toxin portion by a peptide bond. Examples of naturally-occurring proteinaceous toxins that could be incorporated into the immunotoxin of the invention include diphtheria toxin; *Pseudomonas* exotoxin A; ricin and other plant toxins such as abrin, modeccin, volkensin, and viscumin; cholera toxin (produced by *Vibrio cholerae* bacteria); Shiga toxin (produced by various strains of *Shigella* bacteria); the so-called "Shiga-like" toxins (produced by *E. coli* and other enteric bacteria); *Salmonella* heat-

labile enterotoxin; and *E. coli* heat-labile enterotoxin. Non-proteinaceous toxins include known cytotoxic anticancer agents such as doxorubicin, as well as α-emitting radionuclides such as astatine and β-emitting nuclides such as yttrium. An immunotoxin of the invention would be useful for targeting and killing tumor cells which express LCAP on their surfaces.

Also within the invention is an imaging agent in which an LCAP-specific monoclonal antibody, or an LCAP-binding fragment thereof, is linked to a detectable label such as a radionuclide (for example, ^{125}I, ^{131}I, technitium, or indium). Such an imaging agent would be useful for detecting tumors *in situ* by a method including the steps of identifying an animal suspected of having a tumor; introducing the imaging agent into the animal; and detecting (e.g., by radioimaging, using scintigraphy) the presence of the detectable label bound to a tissue (e.g., lung tissue) of the animal, a high level of such label bound to a given site being indicative of a tumor at that site. Using such an imaging method permits a non-invasive determination of the presence, location, or absence of an LCAP-expressing tumor in such animal, which would be particularly useful for monitoring the condition of a patient being treated for a tumor known to express LCAP.

In yet another aspect, the invention features a vaccine for immunizing a human against tumors which express LCAP, which vaccine would include the protein portion of LCAP, or an antigenic fragment thereof, in a pharmaceutically-acceptable carrier. The vaccine would preferentially also include an adjuvant such as Freund's, to enhance the recipient's immune response to the vaccine. Formulation of such a vaccine would be routine to one of ordinary skill in the art, given the disclosures set forth herein.

Detailed Description

The drawings are first briefly described.

Drawings

Fig. 1 is a set of graphs illustrating the results of indirect immunofluorescence of human lung carcinoma cell lines with MAbs DF-L1 and DF-L2. Suspensions of CALU-3, SK-MES, A-549, and ZR-75-1 cells were incubated with MAb DF-L1 (heavy solid line), MAb DF-L2 (dotted line), or an isotype-identical control antibody (thin solid line). After a second incubation with fluorescein-conjugated goat anti-mouse IgG1, the cells were analyzed by flow cytometry.

Fig. 2 is an immunoblot analysis of human tumor cell lines with MAb DF-L1. *A*, human lung carcinomas; *B*, other human tumors. Extracts of the indicated cells were subjected to 3-10% gradient SDS-PAGE. The proteins were then transferred to nitrocellulose paper and monitored for reactivity with MAb DF-L1 and ^{125}I-labeled sheep anti-mouse immunoglobulin. *Kd*, M_r in thousands.

Fig. 3 is a set of photographs showing immunoperoxidase staining of primary human lung carcinomas and normal lung tissue. Formalin-fixed tissue sections were stained with MAb DF-L1 using an avidin-biotin-peroxidase method. *A*, adenocarcinoma; *B*, squamous cell carcinoma; *C*, normal alveolar lining cells; *D*, normal bronchus (*arrow*, terminal web of brush border).

Fig. 4 is an autoradiogram showing immunoprecipitation of [^{3}H]proline-labeled CALU-3 cells. CALU-3 cells were labeled with [^{3}H]proline for 48 h. Cell extracts were subjected to immunoprecipitation with MAb DF-L1, MAb DF-L2, or an IgG1 control MAb. The immunoprecipitate was analyzed by 3-15% SDS-PAGE and autoradiography. *Kd*, M_r in thousands.

Fig. 5 is an immunoblot illustrating the effects of tunicamycin on DF-L1 antigen. CALU-3 cells were grown

in the presence of 10 μg/ml tunicamycin for 24 h and then subjected to immunoblot analysis with MAb DF-L1. *Kd*, M_r in thousands.

Fig. 6 is an immunoblot analysis of human plasma samples analyzed with MAb DF-L1. Plasma specimens (3 μl) from normal subjects (*lanes 1-4*) and patients with lung carcinomas (*lanes 5-8*) were subjected to immunoblot analysis with MAb DF-L1. *Lane 9*, 1 μg of purified CALU-3 antigen. *Kd*, M_r in thousands.

Fig. 7 is a graph showing the reproducibility of LCAP calibrator curves. LCAP calibrators containing 0, 20, 100, and 200 units/ml were assayed in duplicate on 4 consecutive days. Absorbances (ABS) at 490 nm were determined for each calibrator. Open circles, Day 1; closed circles, Day 2; ■, Day 3; ▲, Day 4. *Points*, mean; *bars*, SD.

Fig. 8 is a graph showing the effect of dilution on LCAP levels. Plasma samples from normal subjects and patients with lung cancer were assayed for LCAP levels as a function of dilution. Open circles and squares denote normal subjects; closed circles and squares denote patients with metastatic lung cancer. *Points*, mean; *bars*, SD; *ABS*, absorbances.

Fig. 9 is a histogram illustrating the distribution of LCAP levels in normal subjects. Plasma from normal subjects was assayed, and the number of subjects with LCAP levels within the indicated ranges was plotted as a histogram.

Fig. 10 is a graph showing LCAP levels in patients with lung cancer. Plasma samples from normal subjects and patients with lung cancer were assayed for LCAP levels. A normal cut-off value of 23 units/ml was used. *NSCLC*, non-small cell lung carcinoma; *SCLC*, small cell lung carcinoma.

Fig. 11 is a set of graphs showing serial LCAP levels in patients with lung cancer. Serial LCAP levels of 3 patients treated for lung cancer were determined and compared with clinical course during therapy. *A* and *B*, adenocarcinoma; *C*, small cell carcinoma. *Points*, mean; *bars*, SD.

Fig. 12 is a set of graphs showing LCAP spikes during effective treatment in patients with lung cancer. Serial LCAP levels from a patient with Stage IIIA adenocarcinoma of the lung (12A) and from two patients with limited stage small cell carcinoma of the lung (12B, C) were monitored during chemotherapy and radiotherapy. Clinical evaluations were performed as indicated. *NED*, no evidence of detectable disease. Dotted line = 23 U/ml.

Fig. 13 is a graph showing serial LCAP levels after complete resection of non-small cell carcinoma of the lung. Serial LCAP levels were monitored daily from nine patients with stage I or II non-small cell carcinoma of the lung following complete surgical resection of tumor. Horizontal dotted line = 23 U/ml.

Fig. 14 is a graph of LCAP levels in patients with non-lung malignancies. Dotted line = 23 U/ml.

Fig 15 is a graph illustrating a typical calibration curve obtained with the immunoassay kit of the invention, including points plotted on the curve for two hypothetical test samples.

Immunization of mice with an extract of a primary human lung adenocarcinoma resulted in a panel of monoclonal antibodies, including those produced by the hybridomas designated DF-L1 and DF-L2, which react with extracts from a number of lung and breast carcinoma cell lines. LCAP antigen, which is actually a group of closely-related, high molecular weight glycoproteins, was first identified by subjecting a lung cancer cell line

extract to SDS-PAGE and immunoblotting the gel with the monoclonal antibody DF-L1. An immunoassay utilizing this antibody was subsequently used to detect LCAP in serum samples from human patients, leading to the discovery that LCAP is present in significantly higher levels in samples from patients with lung cancer or certain other types of cancer, than in those from normal, healthy individuals. These studies are described in detail below. Also described below is an immunoassay kit for the detection and quantitation of circulating LCAP, useful for evaluating and monitoring patients with lung cancer, and potentially other types of cancer as well.

I. DETECTION AND CHARACTERIZATION OF LCAP

Materials and Methods

MAb Production. MAbs were generated by techniques analogous to those described previously (Kufe et al., Hybridoma 3:223-232, 1984). Briefly, BALB/c mice were immunized with an extract of a primary human adenocarcinoma of the lung. Mouse spleen cells were fused with P3X63-Ag8.653 myeloma cells (ATCC CRL 1580) and hybridomas cloned three times by limiting dilution in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose, 10% fetal bovine serum, 10% NCTC-109 medium supplement (Sigma), 1% sodium pyruvate, 1% nonessential amino acids, 200 mM L-glutamine, 1% tylosin (Sigma Chemical Co.), and 1% penicillin/streptomycin. Hybridoma cells were injected into pristane-primed BALB/c mice and the MAbs purified from ascites using a Protein A-Sepharose column (BioRad, Richmond, VA). Yields ranged from 0.5-9.0 mg purified antibody/ml ascites fluid.

Culture of Human Tumor Cell Lines. The human lung adenocarcinoma cell line CALU-3 (ATCC HTB 55), the squamous cell carcinoma SK-MES (ATCC HTB 58), and the undifferentiated lung carcinoma A-549 (ATCC CCL 185) were

grown in Eagle's minimal essential medium supplemented with 10% heat-inactivated FBS, 1% sodium pyruvate, 1% nonessential amino acids, and 1% penicillin-streptomycin. The squamous cell carcinoma cell line CALU-1 (ATCC HTB 54) was grown in McCoy's 5A medium (GIBCO) supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin. Human breast and ovarian carcinoma cell lines were maintained as described (Abe et al., Cancer Res. 49:2834-2839, 1989; Friedman et al., Cancer Res. 46:5189-5194, 1986).

ELISAs. Indirect ELISA was performed by coating 96-well polyvinyl microtiter plates with either 20 μg of crude antigen extract or 1 μg of purified antigen in 50 ml PBS. Nonspecific binding sites were blocked with 5% BSA, and the wells were then incubated with MAb for 1 h. After washing, the plates were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Boehringer Mannheim, Indianapolis, IN) for 1 h and washed again. *O*-Phenylene diamine (Sigma Chemical Co.) in 0.1 M citrate buffer, pH 4.5, was used as substrate, and development was monitored by absorbance at 490 nm.

A double-determinant ELISA was performed by coating 96-well microtiter plates with 2.5 μg MAb/well in 0.1 M sodium bicarbonate-0.5 M NaCl buffer at pH 8.7. The wells were blocked with 5% BSA and incubated with antigen in PBS for 1 h. After washing, the wells were incubated with horseradish peroxidase-conjugated MAb in 0.1% Tween 20/PBS for 1 h, washed, developed with *o*-phenylenediamine, and monitored for absorbance at 490 nm. All ELISA incubations were performed at room temperature.

Indirect Immunofluorescence. Cultured cells (1 x 10^6) were washed extensively and incubated with 5 μg Mab for 1 h at 4°C. The cells were then washed and incubated with a 1:100 dilution of fluorescein isothiocyanate conjugated to goat anti-mouse immunoglobulin (Boehringer

Mannheim) for 1 h at 4°C in the presence of 0.2% sodium azide. The cells were again washed extensively and analyzed on a dual-beam fluorescence-activated cell sorter (Coulter, Hialeah, FL).

Immunoblotting. Antigen samples were analyzed by 3-10% gradient SDS-PAGE(12). Gels were then stained with Coomassie blue or PAS(13) or subjected to Western transfer(14). Following Western transfer, the nitrocellulose membrane was blocked with 5% BSA, incubated with primary antibody, washed, and incubated with ^{125}I-labeled sheep anti-mouse immunoglobulin (Amersham, Arlington Heights, IL). The blot was then washed, dried, and exposed with Kodak X-OMAT film.

Immunoperoxidase Staining. Four-μm sections of formalin-fixed paraffin-embedded tissue were stained using an avidin-biotin-peroxidase staining technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Primary antibody was used at a concentration of 0.1 μg/ml. Reactivity was assessed on a 0-3+ visual scale for cytoplasmic and membrane-staining patterns.

Immunoprecipitation. CALU-3 cells were incubated in proline-free medium supplemented with dialyzed FBS for 6 h. The cells were then incubated for 48-72 h with fresh medium with 150 μCi [^{3}H]proline (130 Ci/mmol; Amersham). CALU-3 cells were also incubated in complete medium containing 150 μCi [^{3}H]glucosamine (40 Ci/mmol; Amersham). The cells were lysed with a 1% Nonidet-40/50 mM Tris-HCl lysis buffer (pH 8.0) in the presence of protease inhibitors (5 mM EDTA/20 mM phenylmethylsulfonyl fluoride/20 mM phenanthroline) and the cell extract was centrifuged at 10,000 x g for 10 min. An immunoprecipitation (IP) complex was formed by incubating protein A-conjugated Sepharose CL-4B (Pharmacia) with rabbit anti-mouse immunoglobulin (Organon-Teknika, Malvern, PA). The supernatant of the cell extract was

pre-cleared by incubating with the IP complex and an irrelevant mouse IgG1 MAb. The precleared extract was then incubated overnight at 4°C with MAb followed by incubation for 1 h with IP complex, washed extensively, subjected to SDS-PAGE under reducing conditions, and monitored by fluorography.

Antigen Purification. High molecular weight antigen was purified from spent culture medium of CALU-3 cells. Cells were grown for 3 days past confluence, and spent medium was collected, pooled, centrifuged at 10,000 x g for 20 min to remove debris, and concentrated approximately 30 times in a stirred ultrafiltration cell (Amicon, Danvers, MA) on a YM30 membrane. The concentrated medium was then applied to a Sepharose CL-4B (Pharmacia) sizing column. Fractions were collected and monitored by absorbance at 280 nm and by ELISA. Fractions with antigen activity were collected, pooled, and applied to a MAb affinity column prepared by covalently coupling 4 mg of MAb/ml of CNBr-activated Sepharose CL-4B (Pharmacia). Antigen was eluted with 3 M $MgCl_2$, extensively dialyzed against water, concentrated, lyophilized, and stored at -20°C. Antigen yield was approximately 50%. Purity was monitored by SDS-PAGE followed by Coomassie and PAS staining or immunoblot analysis.

Results

Reactivity with Human Tumor Cells Lines. Immunization of BALB/c mice with an extract of a primary human lung adenocarcinoma resulted in the production of a panel of monoclonal antibodies. Two IgG1 MAbs, designated DF-L1 and DF-L2, were chosen for further study. The specificity of these MAbs was first determined by reactivity against extracts of various human tumor cell lines in an indirect ELISA. Although

the spectrum of reactivity with these antibodies was nearly identical, there were certain quantitative differences. For example, both reacted with all of the lung and breast carcinoma cell lines (Table 1). In contrast, MAb DF-L1 reacted with an extract of OVCAR ovarian carcinoma cells, while there was no detectable reactivity against these cells using MAb DF-L2 (Table 1). Neither MAb reacted with cell lines derived from human leukemias or melanoma (Table 1).

Indirect immunofluorescence was similarly used to determine whether the epitopes recognized by these antibodies are expressed on the surface of lung carcinoma cell lines. As compared to a control antibody, both MAb DF-L1 and DF-L2 demonstrated binding to the CALU-3, SK-MES, and A-549 lung carcinoma cell lines (Fig. 1). However, the patterns of reactivity of the two MAbs against the same cell lines were distinct (Fig. 1). Similar results were obtained with the ZR-75-1 breast cancer cell line (Fig. 1). Taken together with the ELISA data, these findings suggested that MAb DF-L1 and MAb DF-L2 react with distinct epitopes.

Immunoblot analysis of extracts from the lung carcinoma cell lines revealed reactivity with MAb DF-L1 but not with MAb DF-L2. A heterogeneous antigen with an apparent M_r of 350,000-420,000 was detected in CALU-3 cells (Fig. 2A). High molecular weight antigens were similarly detected in SK-MES, CALU-1, and A-549 cell lines, although these reactive species were more homogeneous in size and ranged slightly higher in molecular weight (Fig. 2A). MAb DF-L1 also reacted with a heterogeneous group of high molecular weight antigens in the breast and ovarian carcinoma cell lines (Fig. 2B). In concert with the findings by ELISA, there was no detectable reactivity of this MAb with extracts of the U-937 or HL-60 leukemia lines (Fig. 2B).

Reactivity with Human Tissues. Formalin-fixed paraffin-embedded sections of tumor and normal tissue were evaluated for reactivity with MAbs DF-L1 and DF-L2 using an immunoperoxidase-staining technique. While there was no detectable staining with MAb DF-L2, certain lung cancer specimens reacted with MAb DF-L1 (Table 2). For example, all adenocarcinomas stained intensely with MAb DF-L1 within the cytoplasm and on apical borders. Poorly differentiated areas of the tumor sections stained less intensely than the more highly differentiated areas (Fig. 3A). The percentage of tumor cells reactive with MAb DF-L1 in the adenocarcinoma sections ranged from 40-100%. Squamous cell carcinomas of the lung expressed the DF-L1 epitope to a lesser degree than adenocarcinomas and the staining pattern was distinct. Peripheral, poorly differentiated areas of the squamous cell cancers had rare positive cells. However, a "pavementing" pattern was observed in the central fields, with the more differentiated squamous cells exhibiting intense, membrane-predominant staining (Fig. 3B). Glandular-like structures in mixed histological areas were positive for the epitope. In contrast, sections from five small cell tumors had no detectable MAb DF-L1 reactivity.

Adenocarcinomas of the breast and ovary expressed the DF-L1 epitope to varying degrees (Table 3). In contrast, melanomas, sarcomas, and lymphomas had no detectable staining. Several normal tissues reacted with MAb DF-L1 including kidney and lung. In the kidney, only the distal collecting ducts reacted with the antigen, while the glomeruli were negative. In the lung, normal alveolar lining cells adjacent to tumor tissue stained for this antigen but primarily with an apical pattern (Fig. 3C). Certain areas of the bronchus also were positive for this epitope with staining localized primarily at the terminal web of the brush border of the

ciliated epithelium. Occasional basal cells also showed slight staining (Fig. 3D).

Identification of Reactive Epitopes. The similar patterns of reactivity with MAbs DF-L1 and DF-L2 suggested that these antibodies recognize the same antigen. Indeed, immunoprecipitation of antigens from CALU-3 cells labeled with [^{3}H]proline revealed with same high molecular weight patterns (M_r 350,000-420,000) for both antibodies (Fig. 4). Moreover, in other experiments, unlabeled CALU-3 cell extracts were immunoprecipitated with either MAb DF-L1 or MAb DF-L2. The immunoprecipitates were subjected to immunoblot analysis with MAb DF-L1. Similar bands were identified by MAb DF-L1 regardless of whether the immunoprecipitation was performed with MAb DF-L1 or MAb DF-L2 (data not shown).

Characterization of the epitopes recognized by these antibodies was performed using antigen purified from the culture supernatant of CALU-3 cells. The antigen was fractionated on a Sepharose CL-4B column and then further purified by MAb DF-L1 immunoaffinity. The purified antigen was analyzed by gel electrophoresis. No detectable contaminating proteins or carbohydrates were detected by Coomassie blue and PAS staining (data not shown).

The purified antigen was subjected to treatment with various agents that alter carbohydrate or protein structure. Reactivity of MAbs DF-L1 and DF-L2 was determined by dot immunoblotting. Pronase, but not trypsin, treatment was associated with loss of reactivity for both antibodies (Table 4). In contrast, while alkaline/borohydride and periodate had little effect on MAb DF-L1 reactivity, exposure of the antigen to these agents was associated with loss of MAb DF-L2 binding (Table 4). Similar findings were obtained by double-

determinant ELISA. Neuraminidase had little effect on binding of either MAb, while periodate and alkaline-borohydride treatments predominantly decreased that for MAb DF-L2 (Table 4). Moreover, pronase completely abolished antibody binding and trypsin had only partial inhibitory effects (Table 4). CALU-3 cells were also grown in the presence of tunicamycin to inhibit N-linked glycosylation. However, this agent had no detectable effect on antibody reactivity or electrophoretic mobility of the antigen (Fig. 5). Taken together, these findings suggested that the DF-L1 epitope resides primarily in a peptide structure, while the DF-L2 epitope is comprised, at least in part, of peptide and *O*-linked carbohydrate.

Detection of Circulating Antigen. Plasma samples from normal individuals and patients with lung cancer were monitored by immunoblotting for the presence of this high molecular weight antigen. Low but detectable levels of antigen were present in samples from four normal individuals (Fig. 6). In contrast, reactivity with MAb DF-L1 was clearly greater in plasma samples from four patients with lung cancer (Fig. 6). The electrophoretic mobility of the antigen varied among individuals and up to three reactive species were detectable in certain patients (Fig. 6).

II. DEVELOPMENT AND CHARACTERIZATION OF AN IMMUNOASSAY FOR CIRCULATING LCAP

Materials and Methods

Peroxidase Conjugation. Purified MAb prepared as described above was conjugated to HRP by a modification of published methods (Yoshitake et al., J. Biochem. 92:1413-1418, 1982; Pain et al., J. Immunol. Methods 40:219-223, 1981) using the two heterobifunctional reagents, *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and *N*-succinimidyl-4-(maleimido-

methyl)cyclohexane-α-carboxylate (SMCC) (Sigma Chemical Co., St. Louis, MO). Briefly, SPDP was conjugated to horseradish peroxidase (HRP), and the SPDP was reduced by dithiothreitol. MAbs were conjugated to SMCC in dimethyl formamide. The two protein derivatives were then conjugated through a SPDP-SMCC bridge. Aggregated material was removed by AcA 34 (LKB, Pointet Girard, France) molecular sizing column chromatography.

Assay Format. MAbs (50μg/ml) were adsorbed to 96-well microtiter culture plates in a 0.1 M $NaHCO_3$/0.5 M NaCl buffer (pH 8.5) for 1h. This concentration was chosen following antibody titration to optimize the signal:noise ratio of the high 200-unit/ml calibrator (see "Assay Calibrator"). The solution was aspirated, and the wells were incubated for 1 h with 5% BSA in 0.01 M PBS to block nonspecific protein binding sites on the plastic. The BSA was removed, and samples containing antigen were added to the wells. After a 1-h incubation, the wells were washed 3 to 4 times with a solution of 0.1% Tween 20 in PBS. The plates were then incubated with HRP-conjugated MAb for 1 h and washed again. *O*-Phenylene diamine was used as substrate, and the reaction was stopped after 30 min by the addition of 4 N H_2SO_4. Absorbance was read spectrophotometrically at a wavelength of 490 nm. All steps were carried out at room temperature.

Assay Calibrators. Spent tissue culture medium of the human lung carcinoma cell line CALU-3 was pooled, concentrated, and used as a source of LCAP for calibration of the assay. An arbitrary value of 200 units/ml was assigned to the pooled medium, and appropriate calibrators were prepared by dilution in PBS. Calibration curves of absorbance at 490 nm *versus* units were run for each plate, and the values of unknown

samples were determined by a point-to-point linear interpolation of the calibration curve.

Plasma Samples. Plasma samples from normal subjects were obtained from the American Red Cross Greater Ozarks Blood and Tissue Services Blood Bank in Springfield, MO. Plasma samples were collected from patients at the Dana-Farber Cancer Institute, Boston, MA, according to protocols approved by the Institutional Review Board. Samples were collected in evacuated tubes containing disodium EDTA. Plasma was separated by centrifugation at 100 x g for 15 min, aliquoted, and stored at -70°C.

Results

Development of LCAP Assay

A panel of 10 MAbs was generated on the basis of reactivity with purified LCAP derived from CALU-3 cells. As determined by direct immunoassay, 3 of the 10 MAbs retained reactivity with purified LCAP after conjugation to HRP. Moreover, 4 of the MAbs were found to adsorb reliably to microtiter plates, retaining activity. These MAbs were evaluated in various combinations to detect LCAP levels in solid-phase ELISAs. The five "sandwich" immunoassay combinations that detected soluble LCAP from cultured cell supernatants were then evaluated for optimal circulating LCAP detection with a small panel of plasma samples from lung cancer patients and from normal individuals (Table 5). The combination DF-L1/DF-L1-HRP distinguished cancer patients from normal controls to the greatest extent and was thus chosen for a more comprehensive analysis.

Preparation of LCAP Calibrators

Spent tissue culture medium from CALU-3 human lung adenocarcinoma cells, containing high concentrations of LCAP as determined by Western blot analysis, was pooled and diluted to make arbitrarily-defined calibrator

solutions, ranging from 0 to 200 units/ml. The LCAP assay was quite reproducible, as demonstrated in a series of calibration curves run on different days (Fig. 7). Serial dilutions of plasma samples from normal subjects and from lung cancer patients were evaluated using the DF-L1/DF-L1-HRP assay (Fig. 8). In undiluted plasma, absorbance was at the upper limits of the assay for both normal and cancer samples. However, when the plasma was diluted, absorbance levels rapidly decreased to the baseline signal for normal samples, while those for samples from patients with advanced lung cancer remained elevated until 1/200 or higher dilutions. The optimal dilution at which absorbance levels for normal samples were near baseline while those for cancer samples remained elevated was 1/50, which was therefore chosen as the standard dilution for all subsequent samples.

Assay Characterization

The LCAP assay was optimized for routine use and then characterized for inter- and intraassay variation, antigen recovery, interference effects, and sample handling.

<u>Reproducibility Studies</u>. Intra- and interassay reproducibility were assessed for the calibrators and three serum specimens containing different concentrations of LCAP. The intraassay reproducibility was determined by one individual running the calibrators and serum specimens in replicates of 12, calculating the mean absorbance value for each and determining the percentage of the coefficient of variation. Interassay reproducibility was determined by running the calibrators and serum samples in duplicate by two individuals over seven assay runs. Intraassay coefficients of variation for the calibrators ranged from 2.67 to 5.57% and from 4.14 to 5.74% for the serum samples (Table 6). Interassay coefficients of variation ranged from 4.29 to

9.52% for the calibrators and 4.13 to 7.61% for the samples (Table 7). Thus, the reproducibility of the assay was satisfactory.

Antigen Recovery. Known quantities of LCAP were added to four plasma samples containing baseline levels of endogenous LCAP. These samples were then assayed, and recovery was determined by dividing the observed value by the expected value and multiplying by 100. Recovery ranged from 96.4 to 106.0% of added LCAP (Table 8).

Interference Studies. The ability of the assay to quantitate LCAP in the presence of potentially interfering, circulating substances was investigated. Known quantities of LCAP were added to plasma specimens with baseline levels of endogenous LCAP that contained different levels of bilirubin (up to 22.1 mg/dl), rheumatoid factor (≥1:160), or triglycerides (up to 1026 mg/dl) These substances are frequently elevated in plasma specimens and often interfere, by nonimmunological mechanisms, in the performance of some immunoassays. The specimens were then reassayed in the LCAP assay, and the percentage of recovery was calculated as above (antigen recovery). The data (Table 9) indicated little if any quantitive interference by these substances in the assay.

Freeze/Thaw. To assess the stability of LCAP in samples that had been frozen and thawed, specimens were cycled between freezing (-20°C) and thawing (18°-25°C) 9 times and assayed along with aliquots of these same samples that had only been frozen and thawed once. No apparent effect on LCAP levels was observed through 9 freeze-thaw cycles compared with one cycle, as all sample recoveries were >90% of control.

Matched Serum-Plasma Correlation. Serum and EDTA plasma samples were obtained from 156 volunteer donors and assayed for LCAP levels. The values obtained for the plasma samples were regressed on the serum values. The

correlation coefficient obtained was 0.966, and the slope, 0.979. The overall concordance using 23 units/ml as a reference cut-off (see following section) was 94%.

Distribution of Circulating LCAP Levels in Normal Subjects

Plasma samples from 341 normal blood bank donors were evaluated for LCAP levels (Fig. 9). The mean value of the normals was 7 units/ml (SD ± 8) with a median value of 5 units/ml. The levels ranged from a low of 0 units/ml to a high of 43 units/ml. Thirty-five percent of the samples had LCAP levels of 2 units/ml or less, while only 13.8% of the samples had levels above 15 units/ml. A level of the mean plus 2 SDs, or 23 units/ml, was chosen as a reference cut-off value. Although the mean LCAP level for smokers was slightly higher than for nonsmokers (10 units/ml versus 7 units/ml), the ranges of LCAP levels for smokers (0 to 34 units/ml) and nonsmokers (0 to 43 units/ml) overlapped. Nonetheless, the small difference between LCAP levels in the two groups was statistically significant (Mann-Whitney test, $P = 0.01$).

Distribution of Circulating LCAP Levels in Lung Cancer Patients

A panel of 35 plasmas from patients with metastatic lung cancer was screened to determine circulating LCAP levels. Patients with all four major histological types of lung cancer (adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell carcinoma) were studied. LCAP levels were elevated in 27 of 35 (77.1%) lung cancer patients with a mean of 127 units/ml and a range of 8 units/ml to >1000 units/ml (Fig. 10). The difference in LCAP levels from lung cancer patients and those from normal controls was highly statistically significant ($P < 0.001$). Moreover, LCAP levels were elevated in patients with each histological

type of lung cancer: adenocarcinoma, 16 of 19 (84.2%); squamous cell carcinoma, 4 of 7 (57.1%); other undifferentiated non-small cell carcinoma, 3 of 3 (100%); and small cell carcinoma, 4 of 6 (66.7%).

Serial LCAP Levels

LCAP levels were monitored in selected patients with lung cancer during treatment for metastatic disease. For example, one patient with adenocarcinoma was considered by clinical criteria to have stable disease (Fig. 11A); however, his LCAP levels increased continually for 5 mo prior to clinical documentation of disease progression. Serial LCAP levels from a second patient, also with adenocarcinoma, correlated with a clinically-documented response to therapy over an initial 6-mo period (Fig. 11B). As the patient responded to therapy, LCAP levels decreased, and conversely as disease progressed, LCAP levels increased. Serial levels of LCAP from a patient with small cell carcinoma also paralleled clinical response to therapy (Fig. 11C). Elevated levels of LCAP prior to chemotherapy rapidly decreased to normal and remained below the normal cut-off as the patient entered a complete clinical response.

Non-Lung Cancer Samples

Plasma samples from a small group of patients with cancers other than lung cancer were also screened to determine the specificity of this assay. Elevated levels of LCAP were detected in 58% of patients with pancreatic cancer, 50% of patients with breast cancer, 33% of patients with ovarian cancer, and 20% of patients with prostate cancer. None of the patients with gastric cancer had elevated circulating LCAP levels.

Benign Samples

A small panel of patients with benign lung disease was screened for the determination of LCAP levels. Forty-six percent of patients with chronic obstructive

pulmonary disease (COPD) and 54% of patients with pneumonia had elevated LCAP levels. One patient with tuberculosis had a normal LCAP level. Despite the relatively high percentage of patients with benign disease that had elevated LCAP levels, the means of the LCAP levels were 23.8 and 23.9 units/ml for the COPD and pneumonia patients, respectively, barely above the normal cut-off value.

III. CLINICAL EVALUATION OF IMMUNOASSAY FOR CIRCULATING LCAP

Methods and Materials

Sample Collection and Clinical Evaluation. Plasma samples collected in EDTA-treated tubes or serum samples were obtained from patients with lung cancer, other malignancies, and a variety of benign disorders. Samples from patients with malignancies were collected from patients at the Dana-Farber Cancer Institute and were stored at -70°C until assayed. These samples were collected within protocols approved by the Human Studies Committee. Samples from patients with benign disease were collected at Sinai Hospital (Detroit) and at Asturias General Hospital (Oviedo, Spain).

For the cross sectional and the serial analyses, clinical information was obtained by review of the patients' charts without knowledge of LCAP levels. Only patients with histologically-documented lung cancer were eligible. Patients with a prior history of a non-pulmonary malignancy, except basal cell carcinoma of the skin or *in situ* carcinoma of the cervix, were excluded. For the correlation of serial levels with clinical course, patients were included if more than one sample was available with >30 days between sample collections. Progression was defined as the appearance of any new malignant lesion documented by histologic, clinical, or

radiographic criteria, or a doubling in the largest dimension of any existing tumor. Response was defined as a decrease in size of a measurable lesion by at least 50%, lasting for at least 30 days, and complete response was defined as complete disappearance of known measurable disease. Stable disease was defined as neither regression nor progression of documented disease for at least 60 days. Bone lesions, but not pleural effusions, were considered evaluable, although the appearance of a new malignant effusion was considered as progression.

Percent change in tumor marker (LCAP or CEA) was calculated as:

$$\% \text{ change tumor marker} = \frac{L_f - L_i}{L_i} \times 100$$

in which L_f represents the level at the time of first documented clinical change, and L_i represents the initial level. Increases or decreases of $\geq 25\%$ in serial tumor marker levels were considered significant changes (Tondini et al., Cancer Res. 48:4107-12, 1988). If neither L_f nor L_i was above the cut-off for the respective marker, variations in levels of that marker were not considered significant.

LCAP ELISA. Circulating LCAP levels were assayed using LCAP ELISA kits as described in detail below, using the method set forth below. The kits are manufactured by Terumo Medical Corporation, Elkton, MD. Briefly, monoclonal antibody DF-L1 (50 µg/ml) was adsorbed to 96 well microtiter culture plates (0.1 M $NaHClO_3$/0.5 M NaCl buffer at pH 8.5). The wells were blocked with 5% bovine serum albumin (BSA) in 0.1 M phosphate buffered saline (PBS) for 1 h, washed, and samples containing antigen (1:51) were added to the wells. After a 1 h incubation, the plates were washed and incubated with horseradish peroxidase-conjugated MAb DF-L1 for 1 h. The plates were

developed with o-phenylene diamine and, after 30 min, stopped with 2 N H_2SO_4; adsorbance was determined at a wavelength of 490 nm. LCAP levels were determined by comparison with a curve generated from standards (calibrators) provided in the kit.

CEA Assay. CEA levels were determined according to the manufacturer's instructions using a microparticle enzyme immunoassay (IMx CEA, Abbott, North Chicago, IL). CEA levels were determined on the same freshly thawed samples used for LCAP determinations.

Squamous Cell Carcinoma Antigen Determinations. Squamous Cell Carcinoma antigen (SCC antigen) levels were determined according to the manufacturer's instructions using a microparticle enzyme immunoassay (IMx SCC, Abbott, North Chicago, IL). SCC antigen levels were determined on the same freshly thawed samples used for LCAP determinations.

Statistics. Comparison of LCAP levels from different populations was determined using the Kruskal-Wallis test (single factor analysis of variance by ranks) (15). Comparisons of two assays in the same population were performed by calculating the normal deviate (z) for differences between proportions (Zar, in J. Zar (ed): Biostatistical Analysis, Prentice Hall, Englewood Cliffs, NJ, pp. 296-298, 1974.)

<u>Results</u>

Distribution of circulating LCAP levels in patients with lung cancer. In the study described above, the mean LCAP level in 341 normal subjects was found to be 7±8 U/ml, only 1% having LCAP levels above 35 U/ml. We chose 23 U/ml (mean+2 SD) as a cutoff to distinguish elevated from non-elevated levels. In the present study, 58 (82%), 55 (77%), 53 (75%), and 30 (42%) of 71 patients with metastatic lung cancer had LCAP levels >20, 23, 25, and 40 U/ml, respectively (Table 10). LCAP levels were

elevated above 23 U/ml in 28 of 33 (85%) patients with adenocarcinoma, 11 of 13 (85%) patients with squamous cell carcinoma, and 6 of 9 (67%) patients with large cell carcinoma of the lung. Of 58 patients with all types of non-small cell lung carcinoma (NSCLC), 46 (79%) had LCAP levels >23 U/ml. Furthermore, 9 of 13 (69%) patients with metastatic small cell carcinoma (SCLC) of the lung also had LCAP values >23 U/ml. LCAP levels were significantly elevated in each of these categories when compared to normal controls.

LCAP levels were also significantly elevated in patients with newly diagnosed, untreated primary carcinomas of the lung without evidence of metastases (Table 11). LCAP levels were >23 U/ml in 15 of 29 (52%) and >40 U/ml in 11 of 29 (38%) patients with all histologic subtypes. Elevated levels were observed in 6 of 12 (50%), 3 of 8 (38%), and 1 of 3 (33%) patients with adeno, squamous, and large cell carcinoma of the lung. Ten of 23 (53%) patients with any type of non-small cell carcinoma had LCAP levels >23 U/ml. In addition, of 5 patients with limited stage small cell carcinoma, 4 (80%) had LCAP levels >23 U/ml and 3 (60%) had LCAP levels >40 U/ml.

Comparison of LCAP and CEA levels. LCAP levels in patients with lung cancer were compared with CEA levels in these same patients. The reported distribution of CEA in normal subjects (Abbott Package Insert:IMX:CEA. 1990) is compared with that of LCAP in Table 12. We chose a cut-off of 4 ng/ml, which defines roughly 95% of the normal population (including smokers and non-smokers), for comparison with an LCAP cut-off level of 23 U/ml. While 77% of all patients with metastatic lung cancer had LCAP levels >23 U/ml, only 44 of 71 (62%) had elevated CEA levels (Table 12). This difference is statistically significant (p=0.05).

In all patients with metastatic lung cancer, 36 of 71 (51%) had both markers elevated, and only 11 of 71 (15%) had neither. The markers were discordant in 24 of 71 (34%). Sixteen of these 24 (23% of total 71) had only an elevated LCAP level, and 8 of the 24 (11% of total 71) had only an elevated CEA level. In total, 60 (85%) had either LCAP >23 U/ml or CEA >4 ng/ml (Table 12). Combining the two assays was significantly more sensitive than using CEA alone for all patient with metastatic disease (p>0.05). Combining the two assays also increased the sensitivity compared to LCAP alone, but this increase was not statistically significant. Thus, the two assays complement one another in patients with metastatic lung cancer, although LCAP is generally the more sensitive of the two.

LCAP levels were compared with CEA levels in patients with different sites of disease (Table 13). LCAP levels were more commonly elevated than CEA levels in patients who had any evidence of visceral metastases, including pulmonary, bone, or liver (79% vs. 63%) (Table 13), but the two assays had similar sensitivity in patients without organ involvement (data not shown). Moreover, while the two assays were equally sensitive in patients with liver metastases, LCAP was significantly more sensitive in patients who did not have liver metastases. Of these 62 patients, 49 (79%) had LCAP levels >23 U/ml, while only 36 (58%) had CEA levels >4 ng/ml (p<0.05) (Table 13). Furthermore, combining the two assays increased sensitivity to 85%, which was significantly superior to CEA alone, but not to LCAP alone. Thus, neither assay was very sensitive in patients with minimal metastatic disease (for example, metastases only to regional lymph nodes), and both assays were equally sensitive in patients with liver metastases. However, LCAP levels were more commonly elevated in

patients with distant metastatic disease in whom liver metastases had not yet been detected.

Comparison of LCAP and SCC antigen levels. In patients with squamous cell carcinoma, LCAP levels were compared with SCC antigen. An SCC antigen level of 3 ng/ml was chosen as a cut-off, since previous studies have demonstrated that 95% of a normal population have SCC antigen levels below this level (Body et al., Cancer 65:1552-6, 1990). Only 2 of 13 (15%) patients with squamous cell carcinoma of the lung had SCC antigen levels >3 ng/ml, compared to 77% who had elevated LCAP levels (data not shown) ($p<0.001$).

Correlation of serial LCAP levels with clinical course in patients with lung cancer. Changes in LCAP levels determined in serial samples were correlated with clinical course (Table 14). Overall, serial LCAP levels correlated with clinical evaluation of disease course in 21 of 49 (43%) patients. In contrast, serial CEA levels correlated in only 15 of 49 (31%). LCAP levels increased in 12 of 25 (48%) patients with progressive disease and decreased in 5 of 7 (71%) patients with disease responding to therapy. Combining the two assays improved the correlation with clinical course in 25 patients with progressive disease. In 16 (64%), either serial LCAP or CEA levels increased by >25% above the respective cutoff.

In 4 of 17 (23%) patients whose clinical course remained stable over at least 60 days, LCAP levels did not change by ±25%. Of interest, in the remaining 13 stable patients, LCAP levels increased by >25% in 4 patients who were found to have progressive disease at the next clinical evaluation. These data indicate that in some patients whose disease was perceived to be clinically stable, increasing LCAP level predicted subsequent clinical progression.

In 3 patients who responded to therapy, serial LCAP levels exhibited a spike, defined as a dramatic increase followed by a decrease to, or nearly to, baseline (Fig. 12). One of these patients had Stage IIIA adenocarcinoma and was treated with three cycles of combination chemotherapy followed by radiation therapy to the primary site, resulting in a partial response (Fig. 12A). During the first cycle of chemotherapy, LCAP levels rose from a baseline of 21 U/ml to a peak of 59 U/ml and then fell to levels slightly above 30 U/ml. Unfortunately, this patient died from post-operative complications, so that long term follow-up was unavailable.

Two other patients in whom LCAP spikes were observed had limited stage small cell carcinoma (Fig. 12B, 12C). Both had pretreatment LCAP levels <30 U/ml, and both achieved a partial response with chemotherapy. In both cases, dramatic rises in LCAP levels were observed during adjuvant radiation therapy to the primary site and the CNS, lasting 4 months or longer. LCAP levels returned to baseline following completion of therapy, and the patients were found to be free of detectable disease at that time.

Monitoring of serial LCAP levels post-operatively in patients with primary, non-small cell lung cancer. Serial, daily LCAP levels were monitored in 9 patients with newly diagnosed primary NSCLC who underwent complete resection of tumor (Fig. 13). In 7 of these patients, LCAP levels were elevated prior to complete resection of their tumor. Post-operatively, LCAP levels fell on a daily basis. Four patients had pre-operative levels <50 U/ml, and serial LCAP values fell below the cut-off of 23 U/ml within 2 days. Three other patients had pre-operative levels between 80 and 100 U/ml, and none of these fell within the normal range within 4 post-

operative days. The mean (±SD) circulating half-life, which was calculated during the postoperative decline to baseline, was 4.4±3 days.

LCAP levels in patient with non-lung malignancies and in patients with benign pulmonary disease. Circulating LCAP levels were also studied in patients with metastatic malignancies of non-lung epithelial tissues (Fig. 14). LCAP levels were >23 U/ml in 31 of 104 (30%) patients with colon cancer, 8 of 14 (57%) patients with breast cancer, 26 of 60 (43%) patients with ovarian cancer, 14 of 26 (54%) patients with pancreatic cancer and 3 of 17 (18%) patients with prostate cancer. Of 8 patients with gastric cancer, none had elevated LCAP levels. LCAP levels were also >23 U/ml in 53% (66/125) of patients with benign pulmonary disease (Table 15). Levels ranged as high as 233 U/ml, although generally levels were <50 U/ml. Of note, LCAP levels were >23 U/ml in 18 of 33 (55%) patients with chronic obstructive pulmonary disease (COPD) (range 9-64 U/ml), and in 5 of 11 (45%) patients with chronic bronchitis (range 4-65). Highest LCAP levels in patients without evidence of lung cancer were found in patients with pulmonary complications of rheumatoid arthritis, with levels of 210 and 233 U/ml in 2 patients.

IV. IMMUNOASSAY KIT

Principle of the Test

In this test for circulating LCAP, serum is incubated in microtiter test wells that have been coated with monoclonal anti-LCAP antibody (DF-L1) for one hour at room temperature (18°C-25°C). Unbound antigen is removed by washing the microtiter test wells. Monoclonal anti-LCAP (DF-L1) conjugated with horseradish peroxidase is then added to the test wells for a one hour room temperature incubation. Unreacted conjugate is removed by washing the microtiter test wells. Enzyme substrate

(H_2O_2) and chromophore (ortho-phenylenediamine) are added and allowed to react for thirty minutes at room temperature. The reaction is stopped and the color developed by the addition of 2N H_2SO_4. The absorbance of the calibrators and unknowns is determined in a suitable spectrophotometer at a wavelength of 492 nm. The absorbance is directly proportional to the concentration of LCAP. A point-to-point calibrator curve is constructed by plotting the absorbance vs. the dose of the calibrators and the unknowns are determined by linear interpolation from this graph.

Procedure

A. Sample Collection and Storage

Blood should be drawn using standard venipuncture technique and the serum should be removed as soon as possible. Handle all samples as if capable of transmitting infectious diseases such as hepatitis B virus or HIV (AIDS).

If the test is to be run within 2 days, the aseptic sample can be stored in a refrigerator (2 - 8°C). For a longer delay between collection and assay, the sample should be stored frozen (at least -20°C).

B. Reagents Supplied - Sufficient for 96 Tests

Upon receipt, all reagents should be stored at 2 - 8°C. The 20x Wash Concentrate should be stored at room temperature (18-25°C). Do not mix different lots of individual reagents within an assay.

LCAP TEST WELL STRIPS:

12 strips (1 x 8 wells) in frame. Mouse anti-LCAP (monoclonal) immobilized on microtiter strip wells. Keep pouch tightly closed during storage.

The wells were prepared as follows: purified DF-L1 antibody (15 μg/ml, 0.15 ml/well) in 0.05M aspartic

acid coating buffer, pH 3.8, was coated on micro titer strips (Nunc), followed by post-coating with 1% bovine serum albumin (BSA) in 0.01M phosphate buffer, pH 7.4, and drying under nitrogen.

LCAP CALIBRATORS:

1x0.8 mL each of four (4) concentrations of LCAP (0, 20, 100, and 200 U/ml), supplied ready to use in a buffered solution [0.01 M sodium phosphate, pH 4.0; 0.15 M NaCl; 10% fetal calf serum (GIBCO); and 0.1% sodium azide].

LCAP CONTROLS:

1x0.1mL each of 25 U/ml and 75 U/ml LCAP in human serum with 1% sodium azide.

LCAP SAMPLE DILUENT: 1 x 25 mL 0.01M phosphate, pH 7.4; 0.15M NaCl; 2% normal mouse serum; 0.02% merthiolate.

WASH BUFFER CONCENTRATE (20x):

1 x 50 mL of 0.2M phosphate, pH 7.4; 0.3M NaCl; 0.2% thimerosal; and 2% tween 20 (Sigma).

ANTI-LCAP HRP CONJUGATE:

1 x 20 mL HRP conjugated with anti-LCAP antibody (DF-L1) in a ratio of 0.8 to 1.2 HRP molecules per molecule of antibody, in a 0.05M Hepes-buffered solution (pH 7.4) with 5% normal mouse serum and 0.02% thimerosal.

COLORIMETRIC REAGENT:

1 x 50 mL citrate buffer (pH 4.0) with 0.003% hydrogen peroxide.

OPD REAGENT:

1 x 5 tablets. Each tablet contains 15 mg o-phenylenediamine dihydrochloride and 140 mg excipient. Keep bottle tightly closed. Avoid contact with skin.

STOP REAGENT:

1 x 10 mL 2 N Sulfuric acid. Avoid contact with eyes and skin.

C. Materials Required But Not Supplied

Pipets:	10 μL - to prepare dilution of samples and controls 0.5 μL - to prepare dilution of samples and controls 100 μL - to dispense samples and controls. 50-200 μL multi-channel pipette - to dispense CONJUGATE, COLOR DEVELOPING SOLUTION and STOP REAGENT.
Mixer:	Vortex mixer or equivalent
Plate Washer:	Capable of washing an 8 well strip or 96 well plate.
Microtiter Plate Reader:	A suitable microtiter plate colorimeter or spectrophotometer that can measure absorbance at 492 nm.
Miscellaneous:	Microtubes (for handling volumes of 0.5 mL)

D. Reagent Preparation

1. SAMPLE and CONTROL PREPARATION (1:51): Dilute each sample and control 1:51 with SAMPLE DILUENT by mixing 10 μL of sample or control with 0.5 mL of diluent.
2. WASH BUFFER (20X): Prepare WASH BUFFER by diluting 50 mL with distilled or deionized water to a final volume of 1 liter. Allow any crystals in the concentrate to dissolve at room temperature followed by thorough mixing before dispensing a partial volume. The diluted wash buffer may be stored at room temperature.
3. COLOR DEVELOPING SOLUTION: Prepare sufficient COLOR DEVELOPING SOLUTION <u>fresh</u> 10 minutes prior to use by dissolving one OPD TABLET in 5 mL of COLORIMETRIC REAGENT for every 40 test wells.

E. Assay Protocol

1. Allow all reagents to reach room temperature prior to use. Mix all reagents thoroughly prior to use.
2. Remove the antibody coated plate from the package of LCAP TEST WELL STRIPS. Return unused LCAP TEST WELL STRIPS to the original pouch provided and seal cut end with tape.
3. Dilute all samples and controls 1:51 in tubes (microtubes) by adding 10 μL of sample to 0.5 mL of SAMPLE DILUENT. Vortex briefly to thoroughly mix.
4. Washing: Fill all wells needed for the run with diluted WASH BUFFER. Allow to soak for 15 ± 5 minutes. Aspirate the plate and wash three (3) times with a suitable microtiter plate washer (1 x 8, or 96 well) or washing manifold (8 or 12 channels). Invert the plate and tap on absorbent pad to remove any excess wash solution.
   Important: Precision will be enhanced by removing as much residual liquid as possible.
5. Pipet 100 μL of CALIBRATORS and CONTROLS in duplicate into the bottom of the test wells.
   Note: CALIBRATORS are ready to use: no dilution is necessary.
6. Pipet 100 μL of the diluted samples in duplicate into the bottom of the appropriate wells.
7. Incubate at controlled room temperature (18 to 25°C) for one hour ± 5 minutes. Start timing after addition of last sample.
8. After sample incubation step is complete, repeat Washing Step 4, but do not soak the plate.
9. Using a multi-channel pipet, add 200 μL of ANTI-LCAP-HRP CONJUGATE into the bottom of each well.
10. Incubate at controlled room temperature for one (1) hour ± 5 minutes.

11. Ten (10) minutes prior to the completion of conjugate incubation, prepare COLOR DEVELOPING SOLUTION by adding one OPD TABLET per five (5) mL of COLORIMETRIC REAGENT (Note - this is enough volume for five strips). Allow tablet to completely dissolve then vortex to homogeneity.

12. After conjugate incubation step is complete, repeat Washing Step 4, but do not soak the plate. Important: Precision will be enhanced by removing as much residual liquid as possible.

13. Using a multi-channel pipet with clean tips, dispense 100 μL of COLOR DEVELOPING SOLUTION into each well and incubate at controlled room temperature for thirty (30) ± 2 minutes. Since the timing of the color development step is critical to the precision and accuracy of the results, it is advisable to add the COLOR DEVELOPING SOLUTION at timed intervals and to add the STOP REAGENT at the same intervals as the color development step.

14. With a multi-channel pipet, dispense 50 μL of STOP REAGENT into each test well, using the same intervals as the color development step.

15. Blank reader on 0 unit CALIBRATOR well. Read the plate at 492 nm immediately after adding stop reagent.

<u>Results</u>

Table 16 shows typical data obtained with this assay.

1. Average the absorbance readings for duplicates of calibrators, controls, and unknowns.

2. Plot a point-to-point calibrator curve of mean absorbance versus Units/ml of each calibrator on linear graph paper (see Fig. 15 and Table 16).

3. Derive unknowns from the calibrator curve as illustrated in Table 16. It is advisable to run controls of known values.

NOTE: A correction for specimen dilution is not necessary since the calibrators are provided in ready-to-use form.

Limitations of the Procedure

1. Samples with LCAP concentrations greater than the 200 UNITS/ml CALIBRATOR should be further diluted into the assay range with SAMPLE DILUENT. The obtained value should be multiplied by this dilution factor to obtain the unit value.
2. Although the assay performance has been established between 18° and 25°C, the absorbance of the 200 unit calibrator has been set at 22°C. Ambient temperatures above or below 22°C may affect the absorbance.

Specific Performance Characteristics

A. Dilution Linearity

Three unknown samples were diluted with SAMPLE DILUENT and assayed for LCAP concentration. The 1:1 sample was used to define the expected values on dilution. (Note: Each sample was previously diluted 1:51). The results are shown in Table 17.

B. Recovery

Known quantities of LCAP were added to four diluted specimens. Recovery was calculated by subtracting the endogenous level from the assayed value, dividing by quantity added, and multiplying by 100; results are shown in Table 18.

C. **Precision**

1. Intra-assay Variation. Data for determining the precision of the assay was obtained by assaying each of five human serum samples in replicates of four (Table 19).

2. Inter-assay Variation. The "between" run statistics were obtained by assaying the five serum samples in replicates of four in each of three assays over three days (Table 20).

D. **Specificity**

1. Varying levels of LCAP were added to human serum samples containing, respectively, triglycerides, bilirubin or rheumatoid factor. As shown in Table 21, no detectable interference was observed in samples having up to the indicated levels of these substances.

2. The effect of other tumor markers on the LCAP assay was determined by adding the marker up to the concentration indicated in Table 22. Unspiked serum was assayed to obtain the endogenous LCAP value. The cross reactivity shown in Table 22 was calculated by dividing the apparent LCAP value (corrected for endogenous) by the quantity added and multiplying by 100.

E. **Sensitivity**

The sensitivity of the LCAP assay was determined by running the calibration curve six times, calculating the mean and standard deviations for the calibrators, adding two standard deviations to the mean absorbance for the 0 Unit CALIBRATOR, and interpolating the LCAP value from the same calibrator curve. The LCAP value obtained was consistently less than or equal to 1.60 Units/ml.

<u>Other Embodiments</u>

Other embodiments of the invention are within the claims set forth below. For example, hybridomas producing LCAP-specific monoclonal antibodies may be prepared by immunization of mice or other animals with

extracts from lung carcinoma cells, as described above, or with preparations of purified or semi-purified LCAP. These antibodies may bind to the same or a different determinant or epitope on LCAP as is bound by the MAb produced by the hybridoma DF-L1 (as determined using standard competitive assays). LCAP may be isolated from the membranes of cells (primary or tissue culture) on which it occurs in relative abundance, or from the bodily fluids of lung cancer patients or other individuals with high levels of the antigen, or from the spent medium of any cell line which, like CALU-3, secretes significant amounts of the antigen. The LCAP immunoassay of the invention can utilize any standard immunoassay procedure known to those who practice the art of immunoassays, including but not limited to ELISA, radioimmunoassay, fluoroimmunoassay, luminescent immunoassay, and competitive immunoassay. Where the immunoassay detects LCAP by sandwiching it between two or more antibody molecules, both antibody molecules can be specific for the same type of determinant on LCAP (there being at least two of such determinant type on each molecule of LCAP), or can bind to different types of determinants on the antigen.

The immunotoxin of the invention can be prepared by chemically conjugating a monoclonal antibody specific for LCAP to any of a number of known toxic entities. A typical way of conjugating antibodies to protein toxins is by crosslinking through a disulfide bond (e.g., Chang et al., J. Biol. Chem. 252:1515-1522, 1977) or a heterobifunctional molecule (e.g., Cawley et al., Cell 22:563-570, 1980). See also Stevens et al., U.S. Pat. No. 4,894,227. Alternatively, the immunotoxin can be prepared by expression of a hybrid DNA engineered to encode both the toxin (or a toxic portion thereof) and the antibody (or an LCAP-binding portion thereof), using

technology available to those of ordinary skill in the art of making such hybrids (see, e.g., Murphy, U.S. Pat. No. 4,675,382, and Chaudhary et al., Proc. Natl. Acad. Sci. USA 84:4538-4542, 1987; each of which is herein incorporated by reference). The DNA sequence encoding the LCAP-binding portion of the immunotoxin would be based upon the variable light-chain (V_L) amino acid sequence and the variable heavy-chain (V_H) sequence of an LCAP-specific antibody of the invention; using the method of Bird et al., Science 242:423-426, 1988, a DNA sequence encoding the V_L joined to the V_H by a linker peptide would be constructed and linked to a DNA sequence encoding the protein toxin (or a toxic portion thereof, as taught by, for example, Murphy U.S. Pat. No. 4,675,382). Such manipulations would be routine to one of ordinary skill in the art of genetic engineering, given the disclosures set forth herein. The resulting immunotoxin could be formulated for use as an anti-cancer agent, following procedures standard to the field of pharmacology.

An LCAP-specific monoclonal antibody can alternatively be combined with a detectable label to produce an imaging agent useful for detecting and localizing LCAP-expressing tumors *in vivo*. Methods of attaching such labels to antibodies are well known in the art, and can be readily accomplished without undue experimentation. The potential usefulness of such an agent can be assayed, for example, by implanting LCAP-specific tumor cells into an immunocompromised host (such as a *nude* mouse) and determining whether or not the imaging agent of the invention detectably labels the tumor produced by such implanted cells.

The vaccine of the invention includes the LCAP protein core from which some or all of the carbohydrate has been removed, or an antigenic fragment of the LCAP protein core, dissolved or suspended in an appropriate vehicle for injection into a person. Although LCAP is a

human glycoprotein found (at relatively low levels) in the circulation of most normal individuals and thus is not inherently immunogenic in humans, removing some or all of the carbohydrate from the LCAP protein core uncovers antigenic sites which are hidden in the naturally-occurring glycoprotein, and which therefore can, when injected as a vaccine in a human, induce an immune response. To the extent that these sites are also relatively exposed on certain tumor cells (which due to their altered metabolism relative to normal cells may express an LCAP antigen with a slightly different carbohydrate makeup than LCAP of normal cells), the antibodies so raised would target any such LCAP-expressing tumor cells for attack by the patient's own immune system. Peptide fragments of the LCAP core protein could be produced enzymatically, chemically, or by genetic engineering, using standard methods. For example, DNA encoding the LCAP core protein could be cloned and sequenced using methods similar to those described in Siddiqui et al., Proc. Natl. Acad. Sci. USA 85:2320-2323, 1988; Merlo et al., Cancer Res. 49:6966-6971, 1989; and Abe and Kufe, Biochem. Biophys. Res. Commun. 165:644-649, 1989, and then genetically manipulated to encode and express defined peptide fragments.

Table 1
Reactivity of MAbs DF-L1 and DF-L2 with human tumor cell lines

Tumor cell line	Reactivity* MAb DF-L1	Reactivity* MAbDF-L2
Lung		
CALU-3	+++	+++
SK-MES	++	+++
A-549	+++	+++
CALU-1	++	+++
Breast		
MCF-7	+++	++
ZR-75-1	+++	+++
BT-20	+++	+++
Ovarian		
OVCAR	++	-
OV-S	-	-
OV-D	++	+
Melanoma		
A-374	-	-
Leukemia		
U-937	-	-
HL-60	-	-

*++++, strong; ++, moderate; +, weak; -, no reactivity.

Table 2
Immunoperoxidase staining of lung tumor tissues with MAb DF-L1

Histology	Tumors positive/tested	% cells positive	Pattern* A	Pattern* C
Adenocarcinoma	6/6	40-100	+++	++
Epidermoid	4/4	20-75	+++	++
Small cell	0/5	0	-	-

*A, apical; C, cytoplasmic; +++, intense staining; ++, moderate staining; +, weak staining; -, no staining

Table 3
Immunoperoxidase staining of normal tissues and non-lung tumors with MAb DF-L1

Tissues	Specimens positive/tested	Cell type positive	Pattern* A	Pattern* C
Normal				
Heart	0/2			
Spleen	0/1			
Testis	0/2			
Ovary	0/2			
Cervix	0/1			
Liver	0/3			
Muscle	0/2			
Lymphoid	0/5			
Cartilage	0/2			
Bowel	0/1			
Endothelium	0/5			
Thyroid	2/2	Follicular	+	-
Breast	4/4	Ducts	++	-
Kidney	3/3	Distal ducts	+	-
Stomach	1/2	Lower glands	+	-
Pancreas	2/2	Ducts	++	-
Lung	5/5	Pneumatocytes	++	-
Tumor				
Breast	10/10	Ducts	+++	+++
Ovary	5/5	Mucus-producing	++	++
Melanoma	0/5			
Sarcoma	0/5			
Lymphoma	0/5			

*A, apical; C, cytoplasmic; +++, intense staining; ++, moderate staining; +, weak staining; -, no staining.

Table 4
Analysis of MAb DF-L1 and DF-L2 binding sites

Treatment	DOT BLOT*		ELISA(% of control)	
	DF-L1	DF-L2	DF-L1	DF-L2
None	+	+	100	100
Trypsin	+	+	77	56
Pronase	-	-	3	2
Neuraminidase	+	+	95	91
Periodate	+	-	100	16
NaOA/borohydride	+	-	79	34

* +, positive reactivity; -, no reactivity.

Table 5
Comparison of assay formats
for the detection of circulating LCAP

Capture MAb	Mean LCAP levels with tracer MAb			
	DF-L1-HRP		DF-L3-HRP	
	Normal subjects	Cancer patients	Normal subjects	Cancer patients
DF-L1	7	92	8	65
DF-L2	1	7	6	51
DF-L4	NE**	NE	8	67

* Means units/ml of 5 samples.
** NE: not evaluated.

Table 6
Intraassay reproducibility of LCAP assay

Sample	Mean ± SD at A_{490} (n = 12)	Coefficient of variation(%)	LCAP (units/ml)
0 units/ml	0.05 ± 0.002	3.90	
20 units/ml	0.38 ± 0.020	5.04	
100 units/ml	1.55 ± 0.090	5.57	
200 units/ml	2.64 ± 0.070	2.67	
Specimen 1	1.34 ± 0.060	4.14	85.6
Specimen 2	0.85 ± 0.050	5.74	52.1
Specimen 3	0.46 ± 0.020	4.88	25.5

Table 7
Interassay reproduceability of LCAP assay

Sample	Means ± SD at A_{490} (n = 7)	Coefficient of variation(%)
0 units/ml	0.061 ± 0.006	9.52
20 units/ml	0.343 ± 0.031	8.90
100 units/ml	1.374 ± 0.072	5.25
200 units/ml	2.442 ± 0.105	4.29
Specimen 1	87.8 ± 3.73	4.25
Specimen 2	56.2 ± 2.32	4.13
Specimen 3	27.7 ± 2.11	7.61

Table 8

Recovery of LCAP after addition to plasma

Specimen	Endogenous LCAP (units/ml)	LCAP added (units/ml)	Expected (units/ml)	Observed (units/ml)	Recovery (%)
1	1.6	179.0	180.6	174.1	96.4
2	4.6	141.0	145.6	144.9	99.5
3	7.9	94.0	101.9	100.0	98.0
4	12.6	45.0	57.6	61.0	106.0

Table 9

Interference studies

Substance and concentration	LCAP added (units/ml)	Recovery (%)
Bilirubin (mg/dl)		
16.3	128.3	109.2
19.5	128.3	110.6
3.0	128.3	106.0
13.6	128.3	100.0
22.1	128.3	108.2
Rheumatoid factor		
≥ 1:160	120.0	99.4
≥ 1:160	120.0	104.2
≥ 1:160	120.0	87.2
≥ 1:160	120.0	92.9
≥ 1:160	120.0	98.0
≥ 1:160	120.0	96.9
Triglycerides (mg/dl)		
872	128.3	103.1
1026	128.3	103.4
345	128.3	105.9
426	128.3	106.0
415	128.3	105.0
536	128.3	112.0

Table 10

Distribution of Circulating LCAP Levels in Patients with Metastatic Lung Cancer

No. (%) of Patients with LCAP levels:

Histologic Category	No. Pts.	>15 (U/ml)	>20 (U/ml)	>23 (U/ml)	>25 (U/ml)	>30 (U/ml)	>35 (Um/l)	>40 (U/ml)	Mean LCAP (+SD)
Normal	341	47 (14)	25 (7)	18 (5.3)	15 (4)	8 (2)	4 (1)	1 (<1)	7.2±8
All Lung									
Cancers	71	63 (89)	58 (82)	55 (77)	53 (75)	46 (65)	38 (54)	30 (42)	114.4±249
All NSCLC	58	54 (93)	49 (84)	46 (79)	45 (78)	39 (67)	35 (60)	28 (48)	133.3±272
Adeno	33	31 (94)	29 (88)	28 (85)	27 (82)	23 (70)	22 (66)	19 (58)	172.5±321
Squamous	13	13(100)	11 (85)	11 (85)	10 (77)	8 (62)	6 (46)	3 (10)	105.8±244
Large Cell	9	7 (78)	7 (78)	6 (67)	6 (67)	6 (67)	6 (67)	5 (56)	68.4±85
Misc.									
NSCLC	4	4(100)	3 (75)	3 (75)	3 (75)	3 (75)	2 (50)	2 (50)	35.5±33
SCLC	13	9 (69)	9 (69)	9 (69)	8 (62)	7 (54)	3 (23)	2 (15)	22.8±11.8

Table 11

Distribution of Circulating LCAP Levels in Patients with Primary Lung Cancer

No. (%) of Patients with LCAP levels:

Histologic Category	No. Pts.	>15 (U/ml)	>20 (U/ml)	>23 (U/ml)	>25 (U/ml)	>30 (U/ml)	>35 (Um/l)	>40 (U/ml)	Mean LCAP (+SD)
All Lung Cancers	29	24 (83)	22 (76)	15 (52)	14 (48)	12 (41)	12 (41)	11 (38)	39.3±33
All NSCLC	24	19 (83)	17 (74)	10 (43)	10 (43)	9 (39)	9 (39)	9 (39)	37.3±33
Adeno	12	11 (92)	9 (75)	6 (50)	6 (50)	5 (42)	5 (42)	4 (33)	32.8±17
Squamous	8	5 (63)	5 (63)	3 (38)	3 (38)	3 (38)	3 (38)	3 (38)	41.6±50
Large Cell	3	3(100)	3(100)	1 (33)	1 (33)	1 (33)	1 (33)	1 (33)	43.3±37
SCLC	5	4 (80)	4 (80)	4 (80)	4 (80)	3 (60)	3 (60)	3 (60)	51.6±34

Table 12

Comparison of LCAP and CEA Levels in Patients with Metastatic Lung Cancer

Histologic Category	No. of Pts.	No. (%) of Patients with: LCAP>23 U/ml	CEA>4 ng/ml	Either LCAP>23 U/ml or CEA >4 ng/ml
Normal		(5)	(5)	
All Lung Cancers	71	55 (77)*	44 (62)	60 (85)*
All NSCLC	58	46 (79)	38 (66)	51 (88)*
Adeno	33	28 (85)	25 (76)	29 (88)*
Squamous	13	11 (85)	7 (54)	12 (92)
Large	9	6 (67)	4 (44)	7 (78)
Misc. NSCLC	4	3 (75)	2 (50)	4 (100)
SCLC	13	9 (69)	6 (46)	9 (69)

$p<0.05$ compared to CEA alone.

Table 13

Comparison of LCAP and CEA Levels
in Patients with Metastatic Lung Cancer by Site of Disease

	ANY LUNG BONE OR LIVER METASTASES				NO LIVER METASTASES			
	No. (%) of Patients with:				No. (%) of Patients with:			
Histologic Category	No. Pts.	LCAP Levels >23 (U/ml)	CEA Levels >4 (ng/ml)	Either LCAP>23 U/ml or CEA>4 ng/ml	No. Pts.	LCAP Levels >23 (U/ml)	CEA Levels >4 (ng/ml)	Either LCAP>23 U/ml or CEA>4 ng/ml
All	56	44 (79)**	35 (63)	48 (86)*	62	49 (79)	36 (58)	53 (85)*
All NSC	46	38 (83)**	31 (67)	42 (91)*	52	43 (83)	33 (63)	47 (90)*
Adeno	28	25 (89)	21 (75)	26 (93)**	29	25 (86)	22 (86)	27 (93)*
Squam	10	8 (80)	6 (60)	9 (90)	12	11 (92)	6 (50)	11 (92)
Large Cell	6	4 (67)	3 (50)	5 (83)	8	6 (75)	3 (38)	6 (75)
Misc. NSC	3	2 (67)	1 (33)	3 (100)	4	3 (75)	2 (50)	4 (100)
SCC	10	6 (60)	4 (40)	6 (60)	10	6 (60)	3 (30)	6 (60)

*p<0.05 compared to CEA alone.

**0.08<p<0.05 compared to CEA alone.

Table 14

Correlation of Serial LCAP and CEA Levels with Clinical Course of Disease

		Number (%) of Patients with Change in Antigen Level that Correlates with Clinical Course[1,2]		
Disease Course	No. of Pts.	LCAP	CEA	Either LCAP or CEA
Progression	25	12 (48)	8 (32)	16 (64)
Response	7	5 (71)	4 (57)	5 (71)
Stable	17	4 (23)	3 (16)	1 (6)
All	49	21 (43)	15 (31)	22 (45)

[1]For patients with progressive disease, antigen levels increased by >25%. For patients with responding disease, antigen levels decreased by >25%. For patients with stable disease, antigen levels did not increase or decrease by ±25%.

[2]If antigen level never above cutoff (LCAP>23 U/ml, CEA>4 ng/ml), antigen not considered to correlate regardless of % change.

Table 15

LCAP Levels in Patients with Benign Pulmonary Conditions

Condition	Pts.	≥23 U/ml (%)	(Mean ± SD)	Range
All	125	66 (53)	37.0 ± 40	3.0 - 233.0
Asthma	6	1 (17)	22.3 ± 18	5.0 - 58.0
Bronchiectasis	3	2 (67)	55.0 ± 34	16.0 - 81.0
Bronchitis	13	7 (54)	29.9 ± 17	4.0 - 65.0
COPD	33	18 (55)	27.2 ± 17	9.0 - 64.0
Pneumonia	28	16 (57)	31.6 ± 23	4.9 - 100.0
Pneumothorax	3	1 (33)	54.7 ± 89	3.0 - 158.0
Pulm. Embolus	4	4 (100)	42.5 ± 7	35.0 - 52.0
Tuberculosis	17	8 (47)	31.0 ± 20	4.0 - 78.0
Cystic Fibrosis	4	2 (50)	26.3 ± 15	13.0 - 49.0
Sarcoldosis	3	2 (67)	88.3 ± 89	20.0 - 189.0
Misc.[1]	11	5 (45)	93.9 ± 102	3.0 - 233.0

[1]Includes Acute Respiratory Insufficiency, Alveolitis, Atelectasis, Bronchial Polyp, Empyema, Hemoptysis, Rheumatoid Lung, Silicosis, Pulmonary Edema, Subcutaneous Emphysema.

Table 16

Typical Data Obtained with LCAP Immunoassay Kit

	Code	Absorb.	Mean Absorb.	LCAP Units/ml
0 UNIT CAL	S1	0.000 0.000	0.000	-
20 UNIT CAL	S2	0.288 0.291	0.290	
100 UNIT CAL	S3	1.205 1.229	1.217	-
200 UNIT CAL	S4	2.325 2.362	2.344	
Control Level 1	C1	0.287 0.299	0.293	20.30
Control Level 2	C2	1.492 1.518	1.505	125.57
Unknown #1	U1	0.396 0.404	0.400	29.53
Unknown #2	U2	1.350 1.250	1.300	107.37

Table 17

Dilution	Sample	Expected (U/ml)	Measured (U/ml)	Correlation (R value)
1:1	1	50.8	50.8	0.999
1:2		25.4	25.7	
1:4		12.7	13.2	
1:8		6.4	5.5	
1:1	2	85.8	85.8	1.000
1:2		42.9	42.8	
1:4		21.5	20.8	
1:8		10.7	9.3	
1:1	3	155.0	155.0	0.997
1:2		77.5	85.0	
1:4		38.8	35.0	
1:8		19.4	15.0	

Table 18

Specimen	Endogenous LCAP (U/mL)	Amount Added (U/mL)	Expected (U/mL)	Observed (U/mL)	% Recovery
1	1.6	179.0	180.6	174.1	96.4
2	4.6	141.0	145.6	144.9	99.5
3	7.9	94.0	101.9	100.0	98.0
4	12.6	45.0	57.6	61.0	106.0

Table 19

Sample#	n	Mean Units	Mean Absorbance	S.D.	%C.V.
1	4	10.2	0.152	0.006	3.99
2	4	28.7	0.372	0.011	2.84
3	4	32.6	0.415	0.013	3.08
4	4	49.4	0.601	0.017	2.81
5	4	93.7	1.158	0.028	2.45

Table 20

Sample #	n	Mean Units	S.D.	%C.V.
1	3	10.5	1.33	12.64
2	3	28.3	0.53	1.87
3	3	30.9	1.65	5.34
4	3	48.9	2.05	4.20
5	3	94.8	6.38	6.72

Table 21

INTERFERENCE

Substance	Concentration	Interference (by Concentration)
Triglycerides	924.0 mg/dL	None
Bilirubin	15.0 mg/dL	None
Rheumatoid Factor	1:160	None

Table 22

Marker	[Normal]	[Added]	[LCAP] (U/ml)	Assayed [LCAP] (U/ml)	% Cross Reaction
CEA	3 ng/ml	5 μg/ml	13.4	12.2	0
NSE	22 ng/ml	5 μg/ml	10.1	10.9	0
CA 19-9	40 U/ml	1000 U/ml	13.0	13.8	0
CA 125	35 U/ml	500 U/ml	13.0	16.4	0.7
CA 15-3	22 U/ml	600 U/ml	14.9	148.4	22.2

Claims

1. An essentially purified preparation of human Lung Cancer-associated Protein (LCAP).

2. The preparation of claim 1, wherein said LCAP is extracted from membranes of human cells.

3. The preparation of claim 1, wherein said LCAP is isolated from a bodily fluid of a person.

4. The preparation of claim 3, wherein said bodily fluid is blood.

5. The preparation of claim 1, wherein said LCAP is secreted by a cell cultured *in vitro*.

6. A method of making the preparation of claim 1, said method comprising

providing a population of cells capable of expressing LCAP;

culturing said population of cells in a medium under conditions which permit said population of cells to express said LCAP; and

isolating said LCAP from the membranes of said population of cells or from said medium.

7. The method of claim 6, wherein said population of cells is descended from a CALU-3 cell [American Type Culture Collection (ATCC) accession No. HTB 55].

8. The method of claim 6, wherein said isolating step comprises the step of contacting said membranes, an extract of said membranes, or said medium with an antibody specific for said LCAP, said antibody being affixed to a matrix material.

9. The method of claim 8, wherein said antibody is the monoclonal antibody produced by the hybridoma DF-L1.

10. The method of claim 7, wherein said medium comprises galactosamine.

11. A hybridoma cell which produces an antibody specific for LCAP.

12. The hybridoma cell of claim 11, wherein said hybridoma is DF-L1.

13. The hybridoma cell of claim 11, wherein said antibody binds to the same determinant on LCAP to which the antibody produced by the hybridoma DF-L1 binds.

14. A monoclonal antibody specific for LCAP.

15. The monoclonal antibody of claim 14, wherein said monoclonal antibody is produced by the hybridoma DF-L1.

16. The monoclonal antibody of claim 14, wherein said antibody binds to a determinant on LCAP to which the antibody produced by the hybridoma DF-L1 binds.

17. A method of producing a monoclonal antibody specific for LCAP, said method comprising culturing the hybridoma cell of claim 11 in a medium, and isolating said antibody from said medium.

18. A method of detecting LCAP in a biological sample, said method comprising

contacting said biological sample with an aliquot containing the monoclonal antibody of claim 14; and

detecting immune complex formation between said antibody and a constituent of said biological sample, said immune complex formation being indicative of the presence of LCAP in said biological sample.

19. The method of claim 18, wherein said immune complex formation is detected by ELISA.

20. The method of claim 18, wherein said biological sample is human serum.

21. The method of claim 18, wherein said monoclonal antibody is produced by the hybridoma DF-L1.

22. The method of claim 18, wherein said monoclonal antibody binds to a determinant on LCAP to which the monoclonal antibody produced by the hybridoma DF-L1 binds.

23. The method of claim 18, comprising the additional steps of

providing a control sample containing a standard amount of LCAP;

contacting said control sample with a second aliquot containing said monoclonal antibody; and

comparing the amount of immune complex formation in said biological sample to the amount of immune complex formation in said control sample.

24. An immunoassay kit comprising

a first reagent comprising a first monoclonal antibody specific for LCAP;

a second reagent comprising an enzyme conjugated to a second monoclonal antibody specific for LCAP;

a third reagent comprising a substrate for said enzyme; and

instructions for using said kit.

25. The kit of claim 24, wherein said first and second monoclonal antibodies are specific for the same determinant on LCAP.

26. The kit of claim 25, wherein said first and second monoclonal antibodies are identical.

27. The kit of claim 24, wherein one of said first and second monoclonal antibodies binds to the same determinant on LCAP to which the monoclonal antibody produced by the hybridoma DF-L1 binds.

28. The kit of claim 27, wherein both of said first and second monoclonal antibodies bind to said determinant.

29. The kit of claim 24, wherein said enzyme is horseradish peroxidase and said substrate is hydrogen peroxide.

30. The kit of claim 24, wherein said kit further comprises a fourth reagent comprising LCAP.

31. The kit of claim 30, wherein said fourth reagent is a calibrator or control sample.

32. An immunotoxin comprising an LCAP-specific monoclonal antibody, or an LCAP-binding fragment thereof, conjugated to a toxin molecule.

33. The immunotoxin of claim 32, wherein said toxin molecule is a protein.

34. The immunotoxin of claim 32, wherein said toxin is chemically conjugated to said monoclonal antibody or said LCAP-binding fragment.

35. The immunotoxin of claim 33, wherein said toxin is linked by a peptide bond to said LCAP-binding fragment, and said immunotoxin is produced by expression of a genetically engineered hybrid DNA molecule.

36. An imaging agent comprising an LCAP-specific monoclonal antibody, or an LCAP-binding fragment thereof, linked to a detectable label.

37. The imaging agent of claim 36, wherein said label is a radionuclide.

38. The imaging agent of claim 36, wherein said antibody binds to the determinant on LCAP to which the antibody produced by the hybridoma DF-L1 binds.

39. The imaging agent of claim 36, wherein said antibody is the antibody produced by the hybridoma DF-L1.

40. A method of detecting tumors *in situ*, said method comprising

identifying an animal suspected of having a tumor;

introducing into said animal the imaging agent of claim 36; and

detecting the presence of said detectable label bound to a tissue of said animal.

41. The method of claim 40, wherein said animal is suspected of having a lung tumor, and said tissue is lung tissue.

42. The method of claim 40, wherein said label is a radionuclide and said detection step is accomplished by radioimaging.

43. A vaccine comprising the LCAP core protein, or a peptide fragment thereof, in a pharmaceutically-acceptable carrier.

44. The vaccine of claim 43, wherein said vaccine additionally comprises an adjuvant.

45. A method of immunizing a human, said method comprising introducing into said human the vaccine of claim 43.

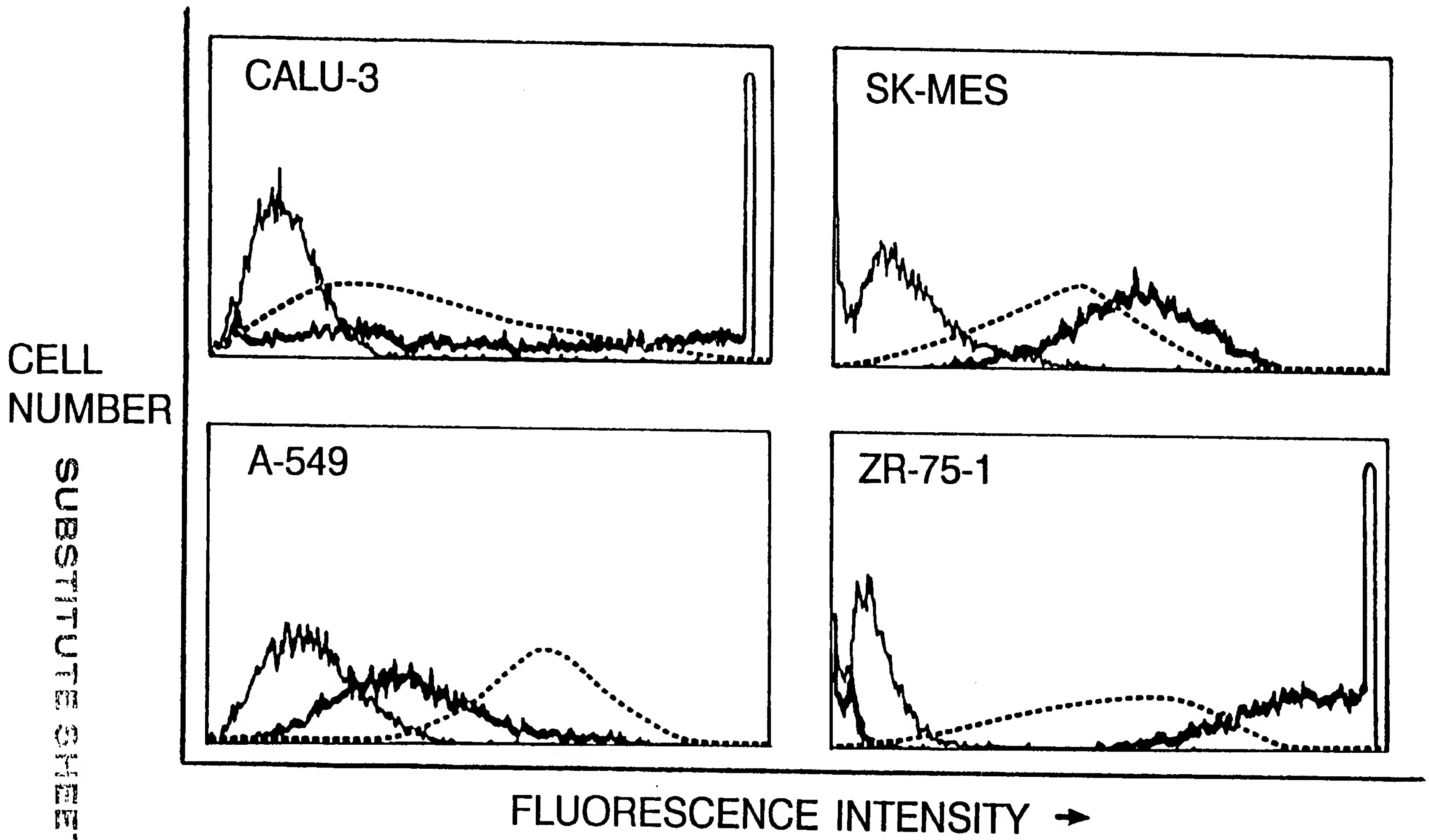
CALU-3
SK-MES
A-549
ZR-75-1
CELL NUMBER
FLUORESCENCE INTENSITY

FIG. 1

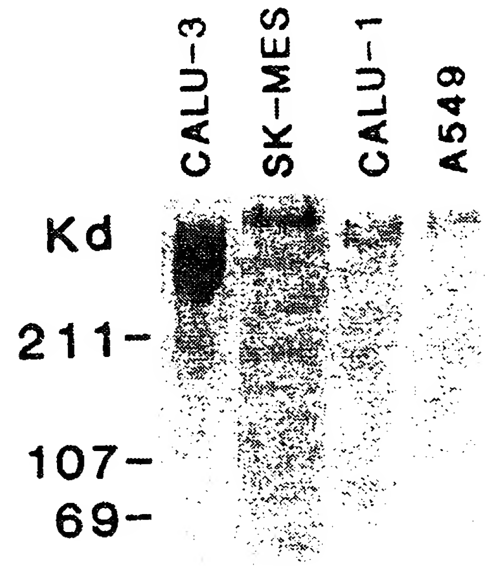

FIG. 2a

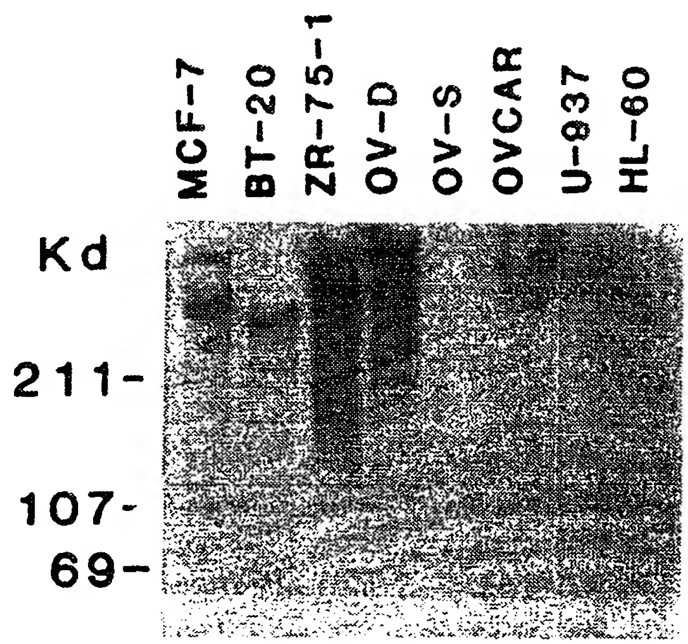

FIG. 2b

SUBSTITUTE SHEET

FIG. 3a

FIG. 3b

SUBSTITUTE SHEET

FIG. 3c

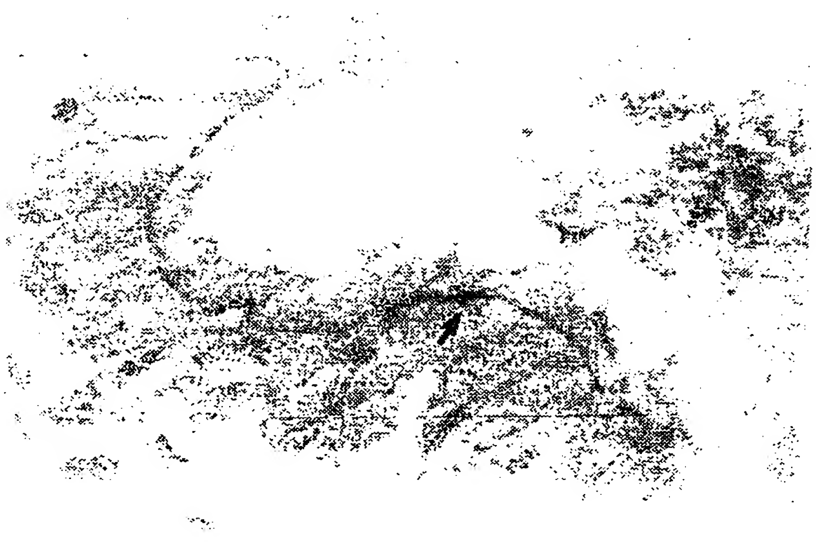

FIG. 3d

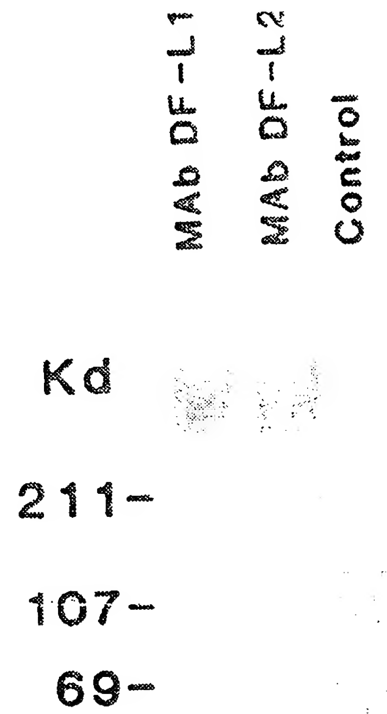
MAb DF-L1
MAb DF-L2
Control
Kd
211-
107-
69-

FIG. 4

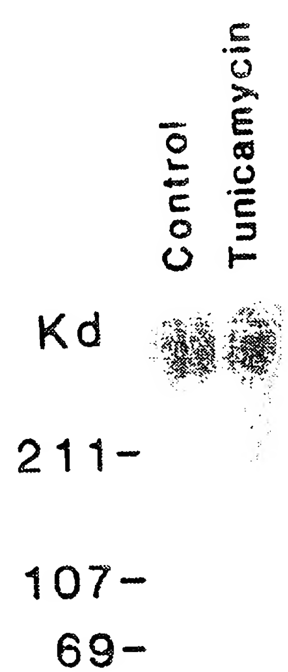
Control
Tunicamycin
Kd
211-
107-
69-

FIG. 5

FIG. 6

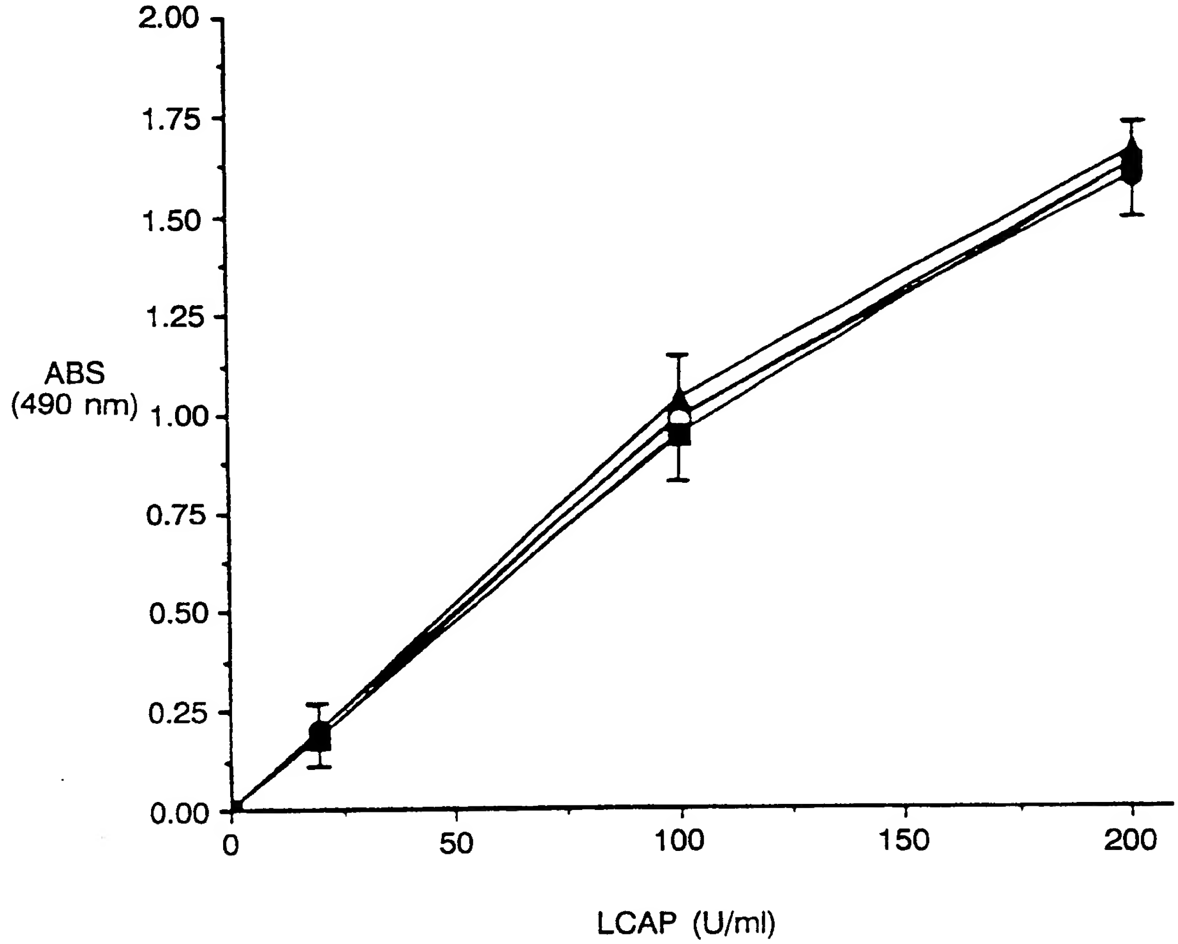
2.00
1.75
1.50
1.25
ABS
(490 nm)
1.00
0.75
0.50
0.25
0.00
0
50
100
150
200
LCAP (U/ml)

FIG. 7

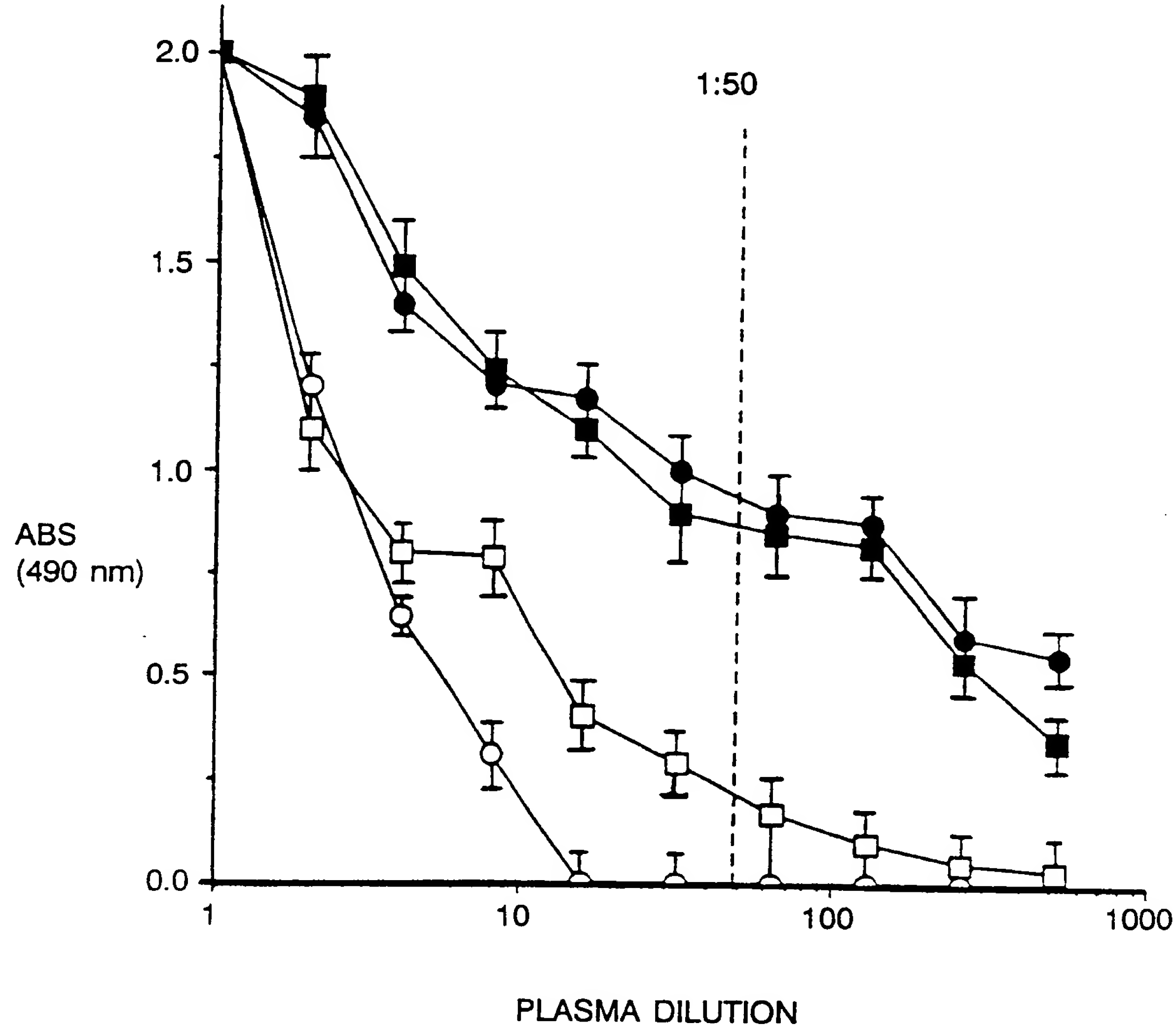

FIG. 8

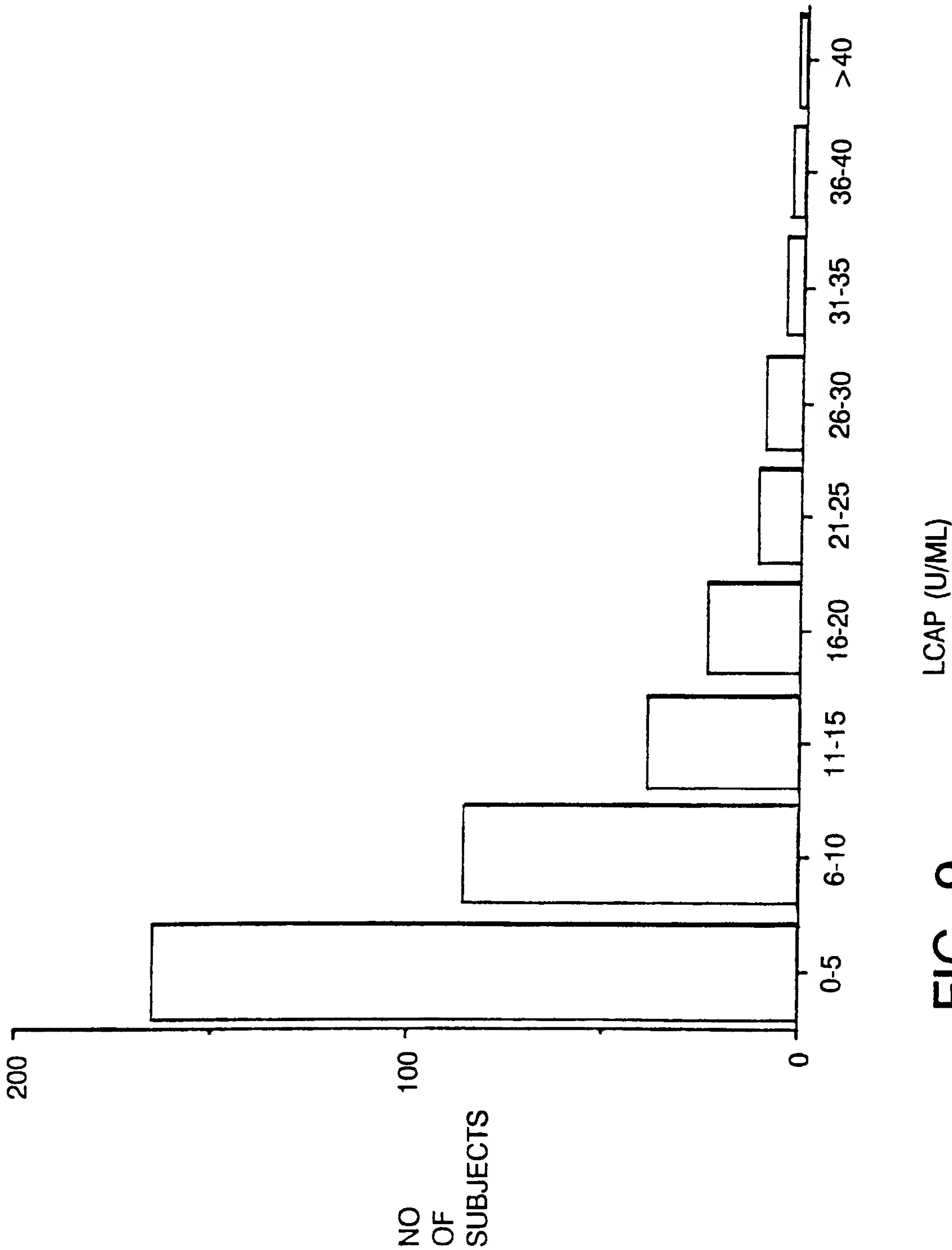

FIG. 9

SUBSTITUTE SHEET

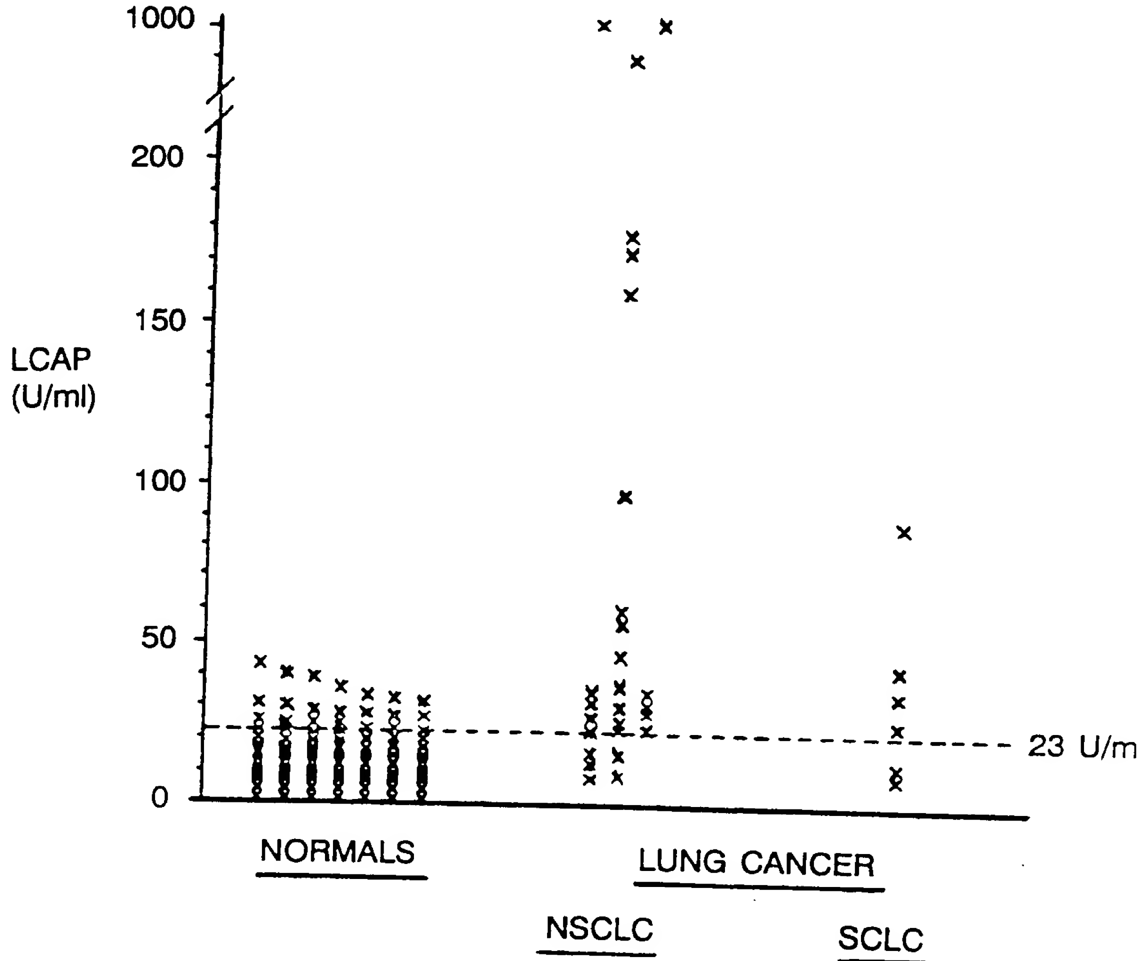
1000
200
150
100
50
0
LCAP
(U/ml)
23 U/ml
NORMALS
LUNG CANCER
NSCLC
SCLC

FIG. 10

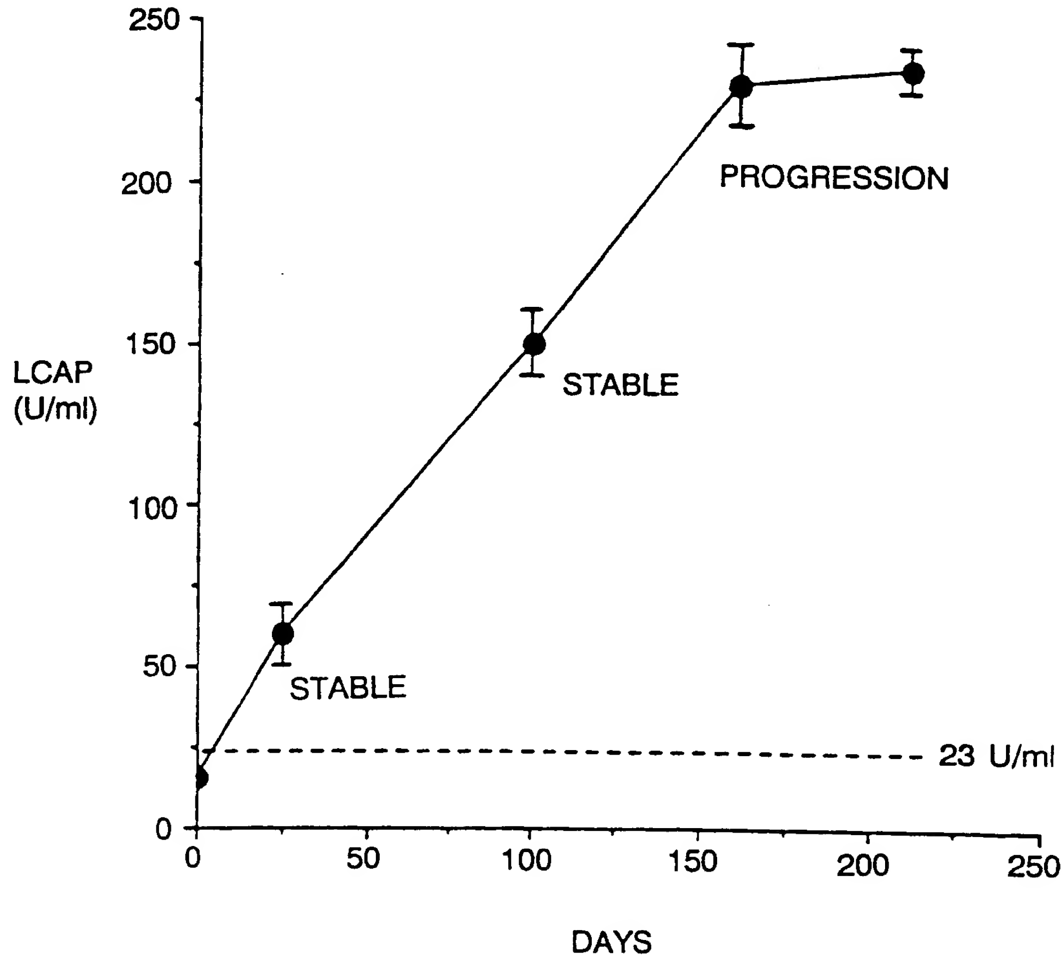

FIG. 11a

SUBSTITUTE SHEET

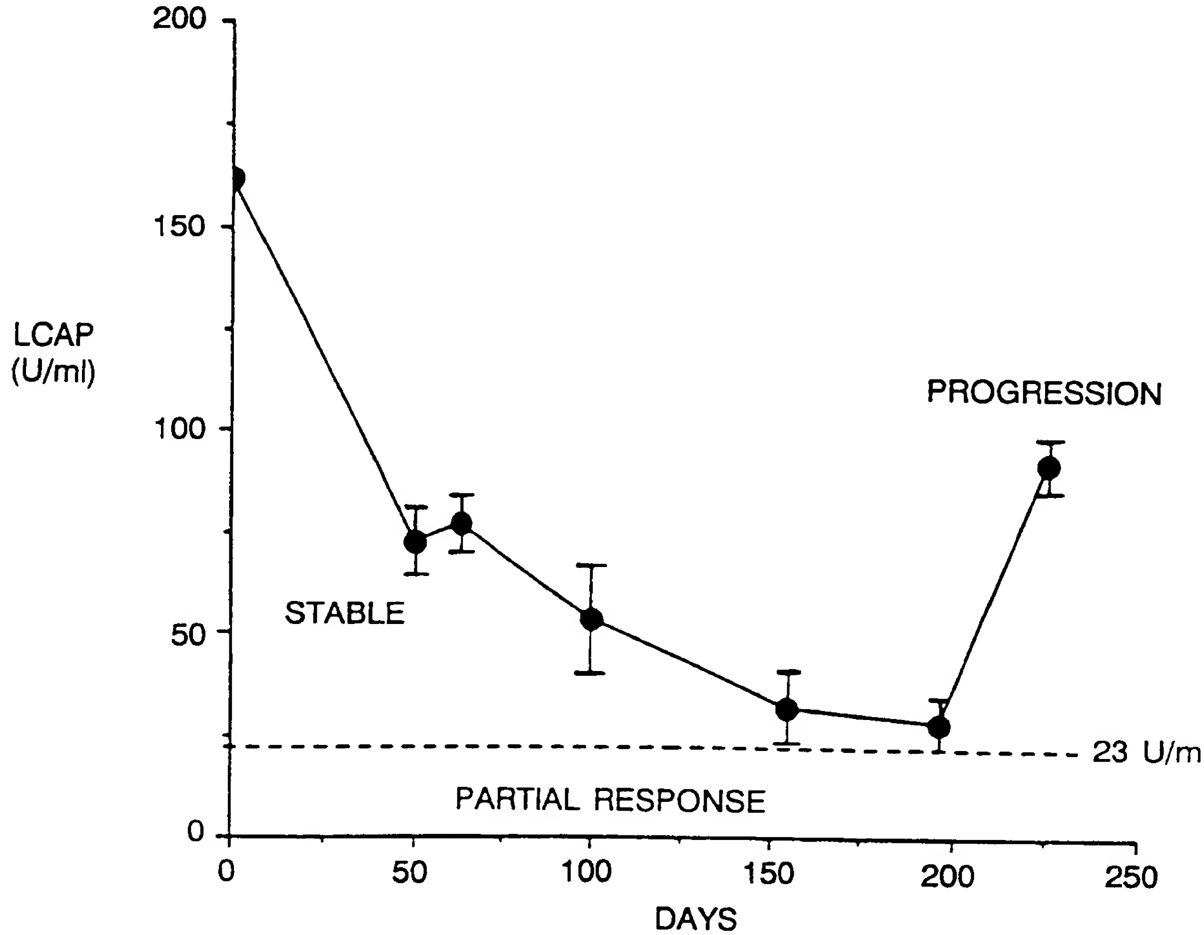
200
150
100
50
0
LCAP
(U/ml)
PROGRESSION
STABLE
23 U/ml
PARTIAL RESPONSE
0
50
100
150
200
250
DAYS

FIG. 11b

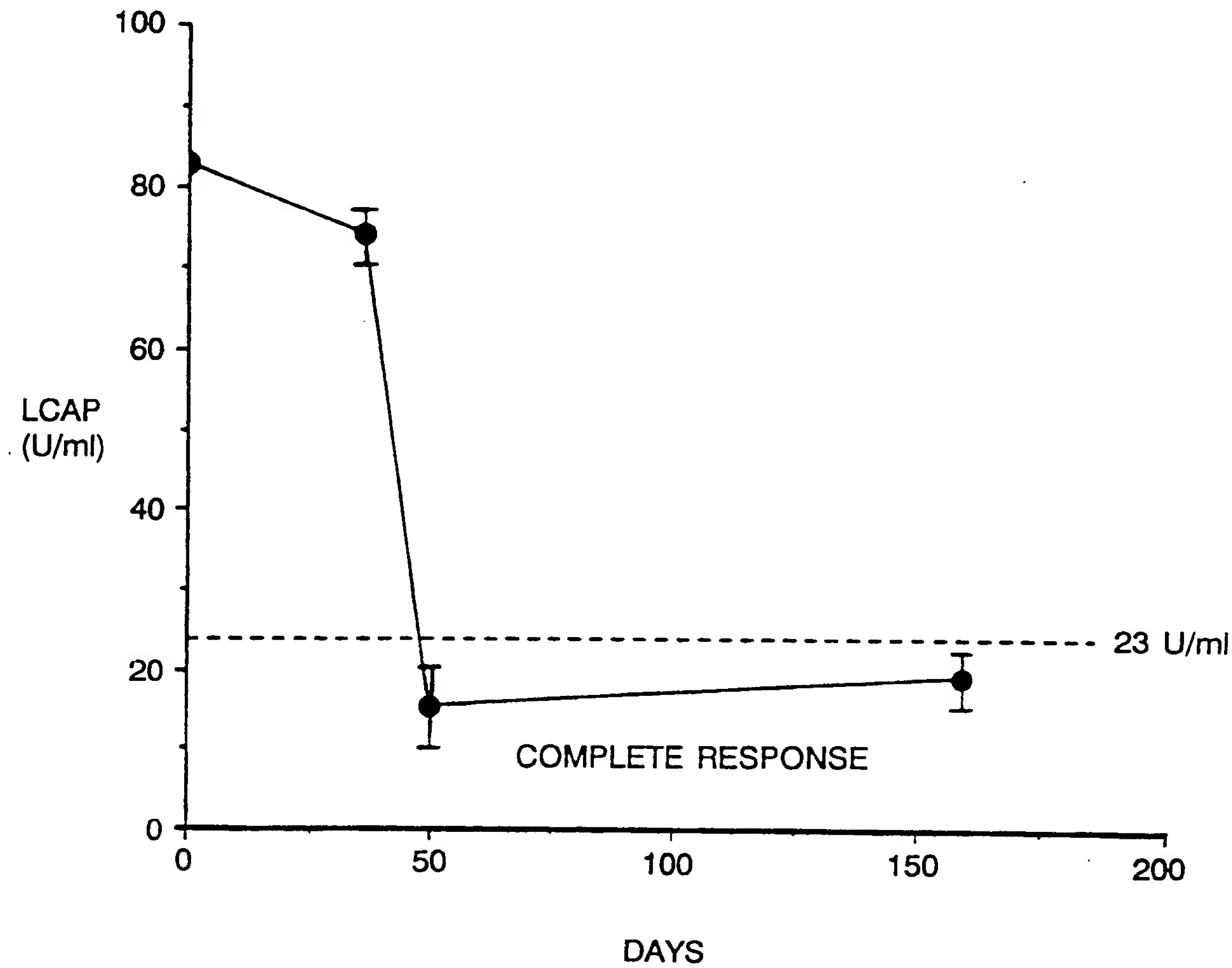
100
80
60
40
20
0
LCAP
(U/ml)
23 U/ml
COMPLETE RESPONSE
0
50
100
150
200
DAYS

FIG. 11c

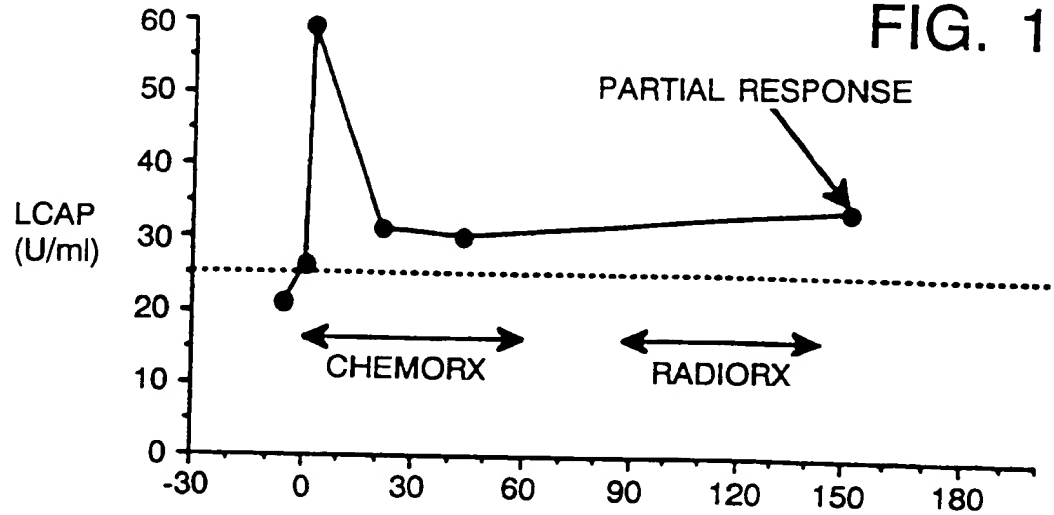

FIG. 12a

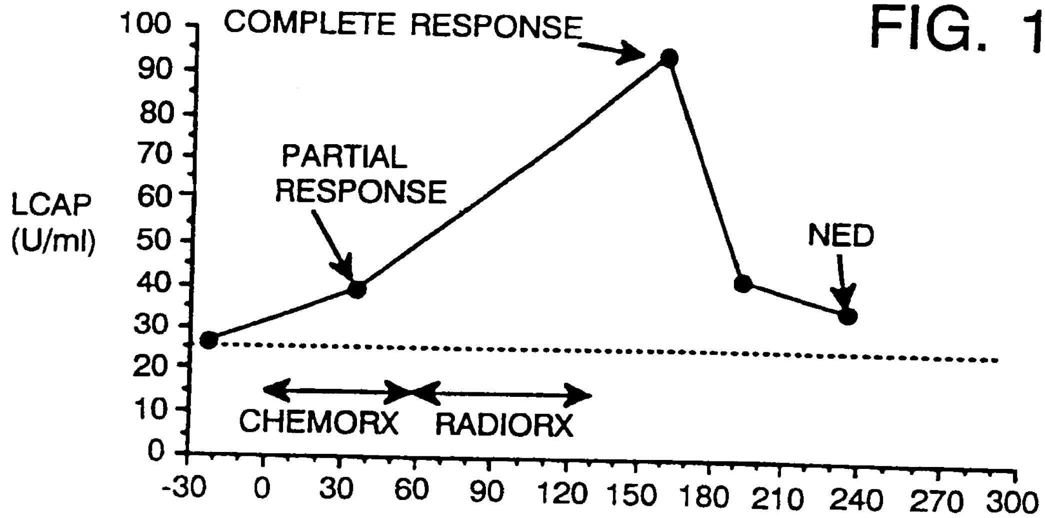

FIG. 12b

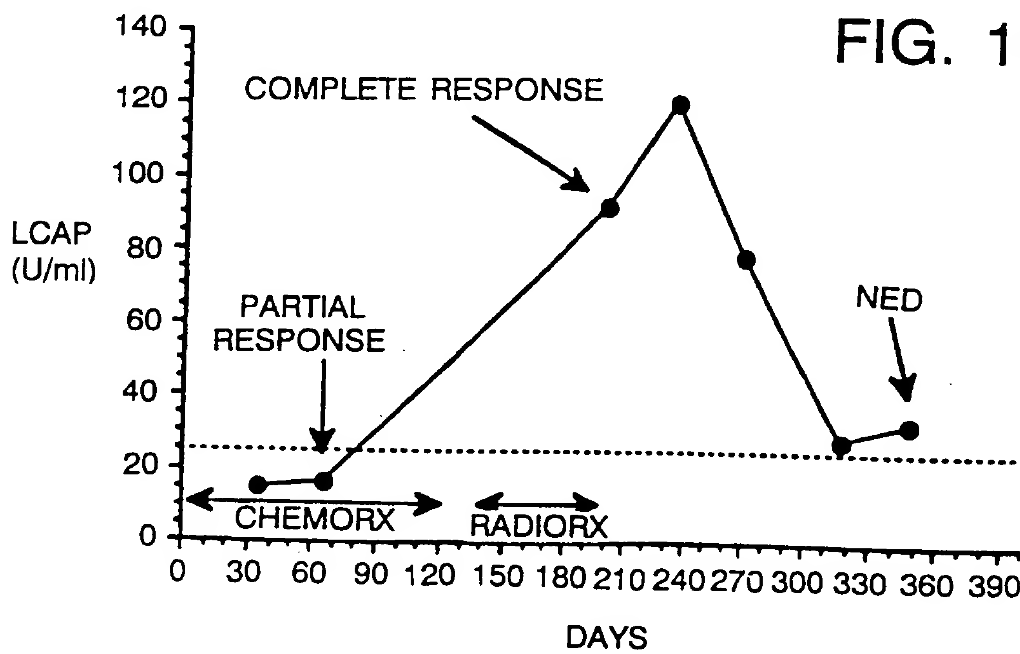

FIG. 12c

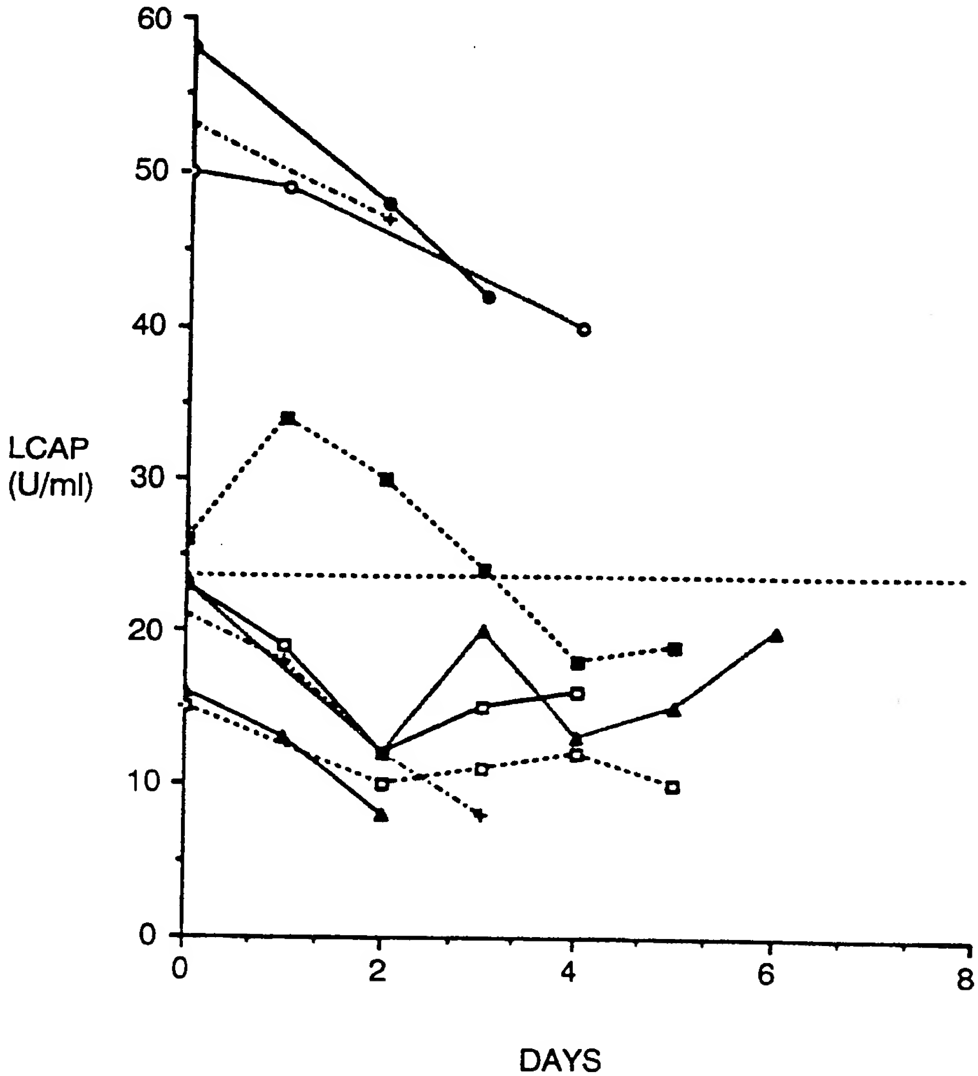

FIG. 13

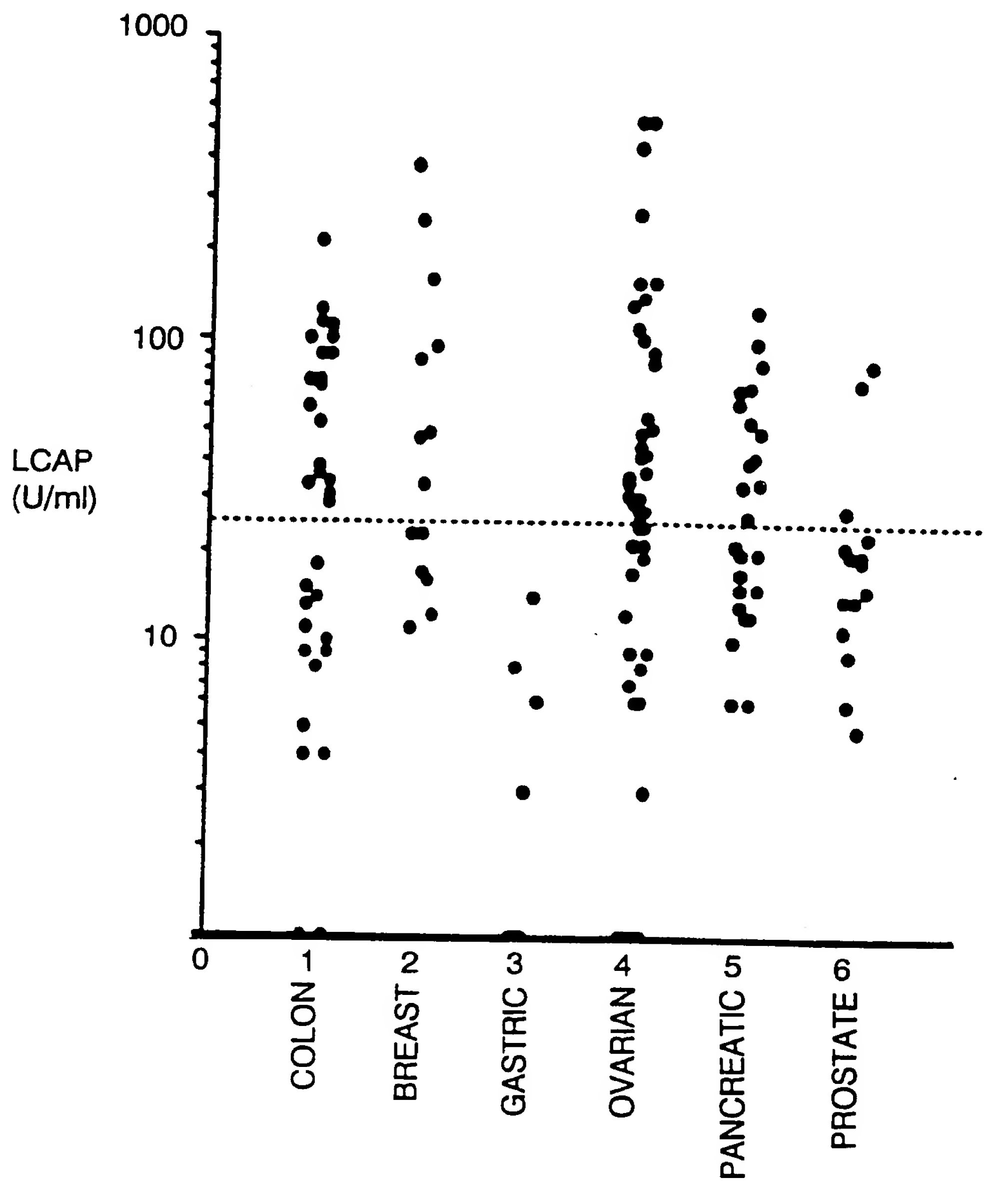
1000
100
10
LCAP
(U/ml)
0
1 COLON
2 BREAST
3 GASTRIC
4 OVARIAN
5 PANCREATIC
6 PROSTATE

FIG.14

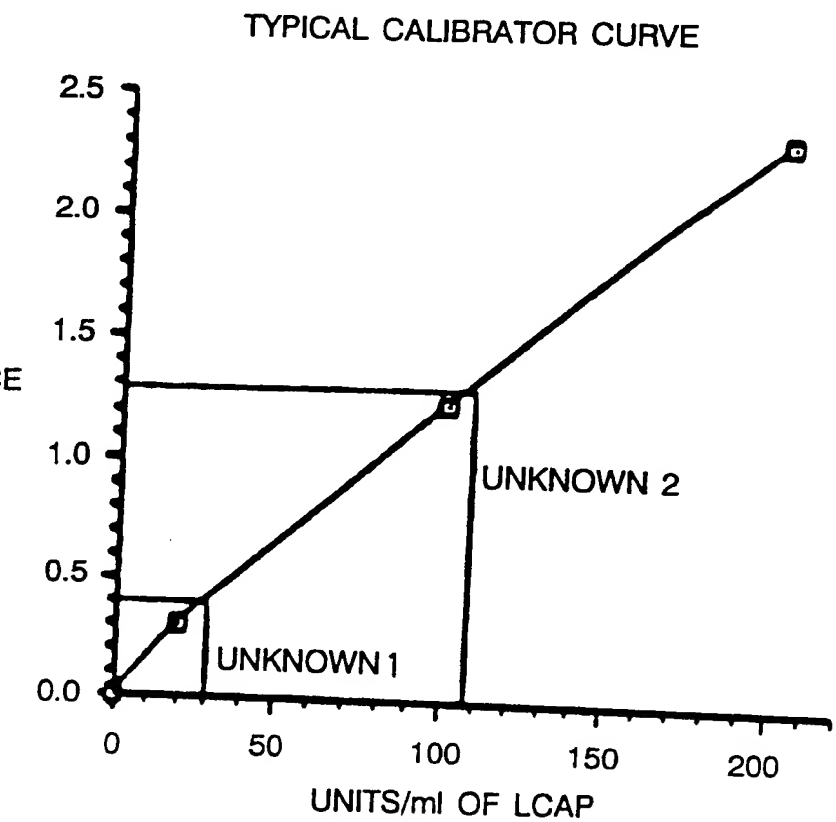
TYPICAL CALIBRATOR CURVE
ABSORBANCE (492nm)
2.5
2.0
1.5
1.0
0.5
0.0
UNKNOWN 1
UNKNOWN 2
0
50
100
150
200
UNITS/ml OF LCAP

FIG. 15

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07585

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC (5): A61K 39/00
U.S. Cl.: 530/300

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
USCl.	530/300;435/7.1, 7.23,7.92

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	CANCER RESEARCH, volume 44, issued May 1984, Rosen et al, "Analysis of Human Small Cell Lung Cancer Differentiation Antigens Using a Panel of Rat Monoclonal Antibodies," pages 2052-2061, see entire document.	1-10 43-45
X Y	CANCER RESEARCH, volume 44, issued November 1984, Okabe et al, "Monoclonal Antibodies to Surface Antigens of Small Cell Carcinoma of the Lung," pages 5273-5278, see entire document.	1-10 43-45
A	CANCER, Volume 65, issued 15 March 1990, Chester et al, "An Analysis of Immunocomplexes for the Detection of the Early Stages of Colon Cancer," pages 1338-1344, see entire document.	1-10,43-45
A	CANCER RESEARCH, volume 50, issued 15 October 1990, Maimonis et al, "Detection and Characterization of a High Molecular Weight Human Lung Carcinoma-associated Glycoprotein," pages 6738-6743, see entire document.	1-10, 43-45

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 January 1992	10 Feb 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Jeffrey Stucker tf

Form PCT/ISA/210 (second sheet) (Rev.11-87)

International Applicat PCT/US91/07585

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers .... because they relate to subject matter [13] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ________ because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers ____________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Please see additional sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

(Telephone Practice) 1-10, 43-45

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

I. Claims 1-10, & 43-45 drawn to a lung cancer-associated protein.
II. Claims 11-13 drawn to a cell line which produces LCAP.
III. Claims 14-16 drawn to a monoclonal antibody against LCAP.
IV. Claim 17 drawn to a method of producing a monoclonal.
V. Claims 18-23 drawn to method of detecting LCAP.
VI. Claims 24-31 drawn to a kit.
VII. Claims 32-35 drawn to an immunotoxin.
VIII. Claims 36-39 drawn to an imaging agent.
IX. Claims 40-42 drawn to a method of detecting tumors.

Group I is drawn to a protein and Group II is drawn a cell line which can produce a protein. Group III is drawn to a monoclonal antibody and Group IV is drawn to a method of making the monoclonal antibody. Group V is a method of detecting LCAP and Group VI is a kit. Group VII is an immunotoxin and Group VIII is drawn to an imaging agent and Group IX is drawn to a method of detecting tumors. The products of Groups I, II, III, VI, VII, and VIII are separate and distinct materials. The methods of Groups IV, V, and IX are separate and distinct methods. The claims of these nine groups are drawn to distinct inventions which are not linked so as to from a single general inventive concept. PCT Rule 13.1 and 13.2 do not provide for multiple products and methods.